


 The logo for the journal Nature, featuring the word "nature" in a white, serif font inside a dark rectangular box.

3 August 1978

Blacks in science: a US national scandal?

ONE of the more striking things noticed by a foreign visitor to a US research laboratory is the extremely low number of black scientists that one meets. Despite more than a decade's concerted efforts to redress racial discrimination and provide educational opportunities for minority groups, the scientific community remains dominated by its white, primarily male, members. And the recent decision of the Supreme Court in the Bakke case, namely that discriminating in favour of minority groups in university enrolment programmes is constitutional but that too rigid an approach is not, has done little to help the situation.

If the position of minority groups in medical schools—the subject of the Bakke decision—is bad, that in science is even bleaker. In a country where blacks make up 15 per cent of the 15 to 21 age group, and 10.7 per cent of the university population, only 4.7 per cent of physical science undergraduates, for example, are black. Even more telling are the figures for PhDs. In a study of 'women and minority PhDs in the 1970s' published recently by the National Research Council, there were only 20 blacks among the 3,166 males receiving doctorates in the physical sciences between 1973 and 1976 (for females the proportion was slightly higher—3 out of 113—but the different totals illustrate another aspect of the problem).

There are no easy solutions. No one seriously believes that the situation can be improved merely by imposing quota restrictions on entrance to university science faculties; nor, for example, by practising reverse discrimination in the award of postgraduate studentships or research grants. For the problem is closely related to broader factors which encourage or rule out access to and interest in scientific

knowledge at a much earlier point in the educational spectrum.

The potential young black scientist, for example, finds the dice loaded against him or her in ways that range from the advice against scientific careers given by teachers and counsellors to the poor quality of mathematics teaching in many of the predominantly black elementary and secondary schools.

From this point of view, a recent decision of the National Science Foundation to award a grant of \$2.8m to the University of Atlanta to set up a Resource Center for Science and Engineering can only be welcomed. The centre will not only make its scientific resources available to a network of mainly black universities and colleges in the south eastern states, but will also involve primary school children from low income neighbourhoods surrounding the university in a 'Saturday science academy'.

The university feels strongly that the NSF should be given sufficient money to establish similar centres throughout the country. Certainly some such major commitment is required if the low level of blacks—indeed of all minority groups—in science is not to remain a national scandal. But the political climate of the US is moving away from the more liberal outlook of the early 1970s. One need only remark the difficulties being experienced by supporters of the equal rights amendment in obtaining ratification from the necessary number of states, or the likely effects of California's proposition 13 on reducing spending on educational programmes for minority groups in general. The present may look bleak. But the future does not look that much brighter. □

Reproductive technology: whose baby?

THE successful birth last week, in the north country town of Oldham, of a healthy baby conceived by *in vitro* fertilisation has been almost universally welcomed and hailed as an important advance in the treatment of certain types of infertility. The baby girl was born to a woman unable to conceive as a result of blocked Fallopian tubes. An ovum was removed surgically and fertilised *in vitro* by her husband's sperm. The fertilised egg was then reimplanted into the mother's uterus where it developed as in a normal pregnancy. The crucial advance in this case was the successful implantation: the first successful *in vitro* fertilisation of a human egg was reported in 1969. Dr Robert Edwards of Cambridge and the Oldham gynaecologist Mr Patrick Steptoe are reported to have made a large number of attempts to implant a fertilised egg back into the mother, but without success, although similar procedures in animals have become almost routine. Providing the correct hormonal treatment to make the uterus receptive is far more

difficult in humans than in animals because of the unpredictability of the human menstrual cycle. This problem has now apparently been overcome and endocrinologists are no doubt looking forward to the promised paper in which the details will be specified.

There can be few, if any, ethical objections to the recent success. The child is both the genetic and legal offspring of her parents, and used in this way the technique is simply another, particularly sophisticated, means of relieving infertility and its attendant unhappiness. However, some of the implications of the foreseeable extensions of this work might be less simply resolved. One proposal is that the fertilised egg might be implanted into a foster mother, in cases where the genetic mother was unable to bear the baby. Whose child would it be? If the practice followed in cases of artificial insemination by a donor other than the husband were followed, the legal parents would be the woman who bore the child, and her husband, if any. To



take legal control the genetic parents would need to adopt the child. *In vitro* fertilisation also offers the chance of determining the sex of a child, once methods for separating male and female sperm are developed. Although this could conceivably lead to an imbalance of the sexes in the population it could also prove beneficial in circumventing the appearance of sex-linked defects in cases where there is no reliable test to detect them *in utero* sufficiently early in pregnancy for abortion to be feasible.

Plans for extending the use of *in vitro* fertilisation in this way must also take into account that it is at present an extremely expensive and specialised procedure, and it is not yet clear that it will ever become routine. The development of better methods for screening fetuses for defects early enough for them to be aborted, if necessary, may in many cases be a more rational use of resources.

An incidental effect of the recent success may be to give the UK Medical Research Council pause to review its policy on funding research in this field. Several years ago it decided not to support work on *in vitro* human fertilisation until sufficient animal work has been done to show that the procedure was safe. This policy was apparently formulated after Edwards and Steptoe applied for a grant, and were

refused, and it still stands although much more animal work has now been done. The lack of applications to do human work has meant that no reappraisal has yet been necessary. Perhaps the MRC will now reconsider its position.

To venture into what some people would still regard as science fiction, the recent success is also a step on the way to much more fundamental manipulation of human beings. Rapid progress in a variety of disparate fields has brought the much-discussed possibility of correcting defective genes by replacing them with a normal copy a little nearer.

Once this sort of intervention is possible the social and political implications could be enormous. On the one hand there may be public pressure to meddle in matters that might be better left alone—in determining the sex of the offspring, for example. And on the other, there is a danger of an emotional response to such meddling that would backfire on the whole of biomedical research. The experience of the nuclear industry, which attempted to forge ahead without taking public opinion with it, has its lessons for biology. The biotechnologists who will surely emerge from our rapidly increasing understanding of molecular biology must take public opinion into careful account. It is not too soon to take these matters seriously. □

Science for the people, or by the people?

Professor Alvin M. Weinberg, Director of the US Institute for Energy Analysis, defends the 'Republic of Science'.

I FIRST encountered Science for the People at a meeting of the American Association for the Advancement of Science in Chicago about eight years ago. The meetings were picketed, and packed, by unruly young people who insisted that they be heard. The Establishment's Science was not responsive to the needs of the people: a revolution in science policy was needed, a revolution that would give to the People a greater voice in determining the course of Science.

Those were stirring times, campus revolutions, Vietnam, and then Watergate; still, Science for the People was regarded by establishment scientists and scientific administrators as a deviation that would go away.

The campus unrest and Vietnam and Watergate have gone away: but Science for the People is widening its influence. As good an example as any is the current excitement over the nutritional etiology of cancer. That nutrition is the most important etiological factor in cancer is regarded as likely by some, but not all, cancer epidemiologists. The question, as far as I can judge, is moot—just as was the viral etiology of cancer about ten years ago. It is a matter that I think deserves careful examination; but this examination must be based on the best judgment of those cancer specialists who have studied the matter. It cannot be settled by popular demand: despite Barry Commoner's recent finding of mutagens in fried (not broiled) hamburgers, it is much too early to conclude that cancer is caused primarily by what we eat.

I could therefore hardly believe my eyes when I saw on national television Senator Robert Dole (the Republican candidate for Vice-President) tax Dr Arthur Upton, director of the National Cancer Institute, for spending too little on the nutritional etiology of cancer. The NCI's annual budget is around \$800m; of this, about 20 per cent goes for environmental etiology of cancer, but only a small fraction of that goes specifically for nutrition and cancer. Senator Dole, fresh from hearings on the subject, proposed that \$200m ought to be spent on the relation between what we eat and cancer.

Dr Upton remained calm and poised despite the Senator's needling—as well he should. After all, the whole war on cancer was itself an example of Science for the People. It was good politics to launch a war on cancer; and as Robert Marston pointed out in his *Nature* review of Rettig's *Cancer Crusade: The Story of the National Cancer Act of 1971*, the war was launched in no small measure because Ann Landers, adviser of the *lowlorn*, had urged one million of her readers to write to Washington about cancer.

In a democracy the directions of scientific research must in some degree respond to the will of the People. The scientists, who, after all, spend public money, cannot fairly object to the public setting the *ends* of scientific research. If the public deems a cure for cancer, or solar energy, or the environment, to be important, then that public has a right to support scientific effort aimed at achieving these goals. To be sure, in many cases, the public's judgment as to whether a given end will yield to scientific inquiry may not agree with the bulk of the scientific community's views. A field must be ripe for exploitation, to borrow jargon from the scholarly debate on priorities, if it is to merit strong support—and the public is generally less competent than is the scientific establishment to make this judgment. The public therefore runs the danger of wasting money—but, so to speak, it is the public's money: the public can choose to spend it wisely or foolishly.

Though the public has a right to establish the ends of science, it does not have the right to designate the means. I have no trouble with the public, through politics, deciding we ought to spend more on cancer or on solar energy. I believe science is endangered if politicians go further and specify how to attack these problems—for example, by directing scientists to spend on nutritional etiology rather than on genetic or viral etiology of cancer, or on the solar satellite rather than on biomass.

What is at issue is the integrity of Michael Polanyi's Republic of Science—the complex institutional structure with its intricate checks and balances that maintains Science as a responsible undertaking. As the political republic encroaches on the scientific republic, it seems inevitable that the latter's integrity is diminished. Science for the People may waste money; Science by the People could cause the whole scientific edifice to crumble. □

US proposes exemptions from DNA guidelines

CERTAIN experiments using recombinant DNA techniques will be exempted from regulatory guidelines, according to proposals published in Washington last week, and the physical and biological containment requirements for a number of experiments will be lowered. These recommendations come in the light of revised estimates about the danger of experiments using disabled host-vector systems. The proposals also suggest that public representatives should be included on local biosafety committees.

The revisions have been prepared by Dr Donald Fredrickson, director of the National Institutes of Health, following lengthy discussion of the present guidelines which have been in force since June 1976. The new guidelines would cover all research carried out by institutions receiving NIH funds for recombinant DNA research, not merely that which is supported by NIH.

The guidelines were published in the *Federal Register* by the Department of Health, Education and Welfare on 28 July, and will be open for public discussion for 60 days. This includes a public hearing next month before a departmental review committee chaired by the department's general counsel, Mr Peter Libassi.

Health Secretary Mr Joseph A. Califano has asked in particular for comments on proposed mechanisms for administering and further revising the guidelines. After the public discussion, the final version of the guidelines will be prepared by Dr Fredrickson and promulgated within 45 days.

In an introduction to the proposed revisions, Dr Fredrickson says it is five years since concern arose about the possible hazards of laboratory experiments using recombinant DNA techniques. During this period no evidence has come to light of a product created by these techniques that has been harmful to man or the environment. "At the very least, there is growing sentiment that the burden of proof is shifting towards those who would restrict recombinant DNA research," Dr Fredrickson says.

Increased confidence about the use of modified host-vector systems has led to a recommendation for changes in required containment levels for certain types of experiments. For example, shotgun experiments involving birds and mammals other than primates, originally requiring P4/EK2 or P3/EK3 containment in the 1976 guidelines, would now require only P2/EK2.

One aspect of the proposed revisions that seems destined to be a target of controversy is the proposal to exempt entirely from the guidelines certain

'The burden of proof is shifting towards those who would restrict recombinant DNA research'

—Dr Donald Fredrickson

categories of experiments. These categories cover experiments using:

- "naked" recombinant DNA molecules which are not in organisms or viruses, and which are considered "extremely unlikely to be hazardous under experimental conditions";
- recombinant DNA molecules which consist entirely of DNA segments from a single non-chromosomal or viral source;
- recombinant DNA molecules made entirely from the DNA of a single organism, including the plasmids, viruses, mitochondria or chloroplasts indigenous to that organism, when propagated only in that organism;
- certain specified recombinant DNA molecules that consist entirely of DNA segments from different species that exchange DNA by known physiological processes. A list of such species would be prepared and periodically revised by the director of NIH (see below);
- any other classes of recombinant DNA molecules which the director of NIH, on the recommendation of his advisory committee, considers "do not present a significant risk to health or the environment".

The proposed guidelines offer a revised definition of recombinant DNA molecules as being either molecules which are constructed outside living cells by joining natural or synthetic DNA segments to DNA molecules that can replicate in a living cell, or DNA molecules that result from the replication of such molecules.

One major change is the decision to lift a previous ban on the use of

Proposed exemptions

Any recombinant DNA molecules to be propagated in species listed here, if the DNA is composed entirely of DNA segments from the same list of organisms, are exempted from control. This exemption acknowledges that these bacteria exchange DNA in nature.

Escherichia species
Edwardsiella species
Citrobacter species (including *Levinea*)
Salmonella species (including *Arizona*)
Shigella species
Klebsiella species
Enterobacter species
Hafnia species
Serratia species
Erwinia species (including *Pectobacterium*)
Pseudomonas species
Rhizobium species
Acinetobacter calcoaceticus
Agrobacterium tumefaciens
Rhodospseudomonas sphaeroides

Vesicular Stomatitis Virus (VSV) and of oncogenic viruses classified by the National Cancer Institute as "moderate risk", and to allow such experiments under specified conditions.

Dr Fredrickson's reasoning behind this is that recombinant DNA experiments with pieces of these viruses cloned in *E. coli* K-12 pose no more risk, and actually appear to pose less risk, than work with the whole infectious virus itself.

With regard to physical containment levels, in addition to introducing a measure of flexibility in the choice of containment equipment, Dr Fredrickson also proposes that pipetting by mouth, already prohibited in categories P2, P3 and P4, should also be prohibited in category P1.

Suggestions are made about the activities of local biosafety review committees (re-named from local biohazard committees). For example, although NIH approval would still be required before new recombinant DNA experiments could go ahead, the local committee could approve subsequent extensions.

In view of the increased responsibility of such committees, Dr Fredrickson suggests adding a provision that "no LBC may consist entirely of persons who are officers, employees or agents of, or are otherwise associated with, the institution, apart from their membership of the LBC."

Other requirements for membership are not laid down, although the proposals suggest that "membership should include individuals from disciplines relevant to recombinant DNA technology, biological safety and engineering"; that "at least one member be a non-doctoral individual from a laboratory technical staff"; and that the LBC include "members knowledgeable about such matters as applicable law, standards of professional conduct and practice, community attitudes, and the environment".

The new proposals also enable the private sector to register voluntarily its recombinant DNA activities with the NIH. Other services, including consulting services and the certification of host-vector systems, would be provided, and the service would be accompanied by protection of proprietary data.

Finally Dr Fredrickson says that "while it is true that other techniques in genetic research, such as cell fusion and chromosome transfer, may result in the formation of recombinant molecules, I do not believe at this time we should mandate or extend the guidelines to research in these areas."

David Dickson

US plays hide-and-seek with Soviets on missile sites

David Dickson describes a new plan for nuclear deployment

CLAUSEWITZ would no doubt have admired the latest move by US defence officials, who are considering plans for a giant \$30 billion "shell and pea" game in which the Soviet Union, if it wants to destroy a particular nuclear missile, would have to guess which of 20 launching holes contained the missile.

The plan, in which the silos would be three to five miles apart, is currently enjoying high favour in Washington military circles as a method for deploying the next generation of inter-continental ballistic missiles, known as MX (for missile experimental). Soviet missiles are now reported to have an accuracy of 600 feet, and it is claimed that such a shell game would avoid the consequent vulnerability of fixed launchers, without significantly increasing the number of missiles deployed.

However there are indications that plans to proceed with what is officially known as a Multiple Aim Point (MAP) deployment scheme could effect the progress of the current Strategic Arms Limitations Talks (SALT 2), now taking place between the US and the USSR in Geneva, since the Soviet Union is concerned over whether the scheme could be adequately verified.

An earlier idea of deploying the MX missiles in a system of tunnels 12 to 15 miles long, along which the missiles could be moved as desired, has now been abandoned following criticism from both within and outside the Defense Department (including a study chaired by the President's science adviser, Dr Frank Press) of the costs involved, and the fear that such a scheme would remain vulnerable to Soviet attack.

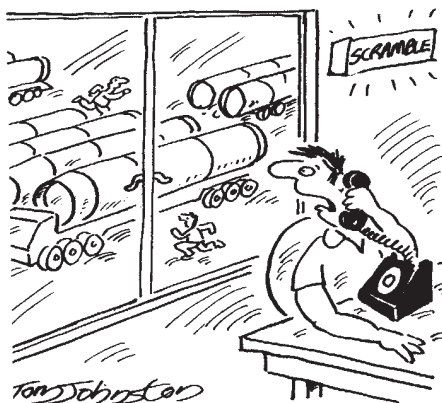
However evidence that the USSR has considerably closed the technological gap with the US since the original SALT agreement of 1972 is being used by Defense Department chiefs, backed by powerful industrial interests, as an opportunity to gather support for the shell game idea. In his first press conference as chairman of the joint chief of staffs, for example, Air Force General David C. Jones said last week that this was "the most attractive way" of protecting land-based missiles.

A number of different variations of the MAP scheme have been closely studied in order to determine the most effective. In addition to the now-discarded tunnel scheme, three others were studied involving the transportation of missiles between different launching sites: one scheme using vertical shelters, a second using

cheaper, but more vulnerable, horizontal shelters, and a third launching the missile from under a pool of water.

The system thought to be most favoured by the Air Force is that involving vertical shelters. The missile would be transported and stored within a canister, and would be launched from the canister using compressed gas before its first stage engine was fired.

The canister would be moved periodically between 20 and 25 different vertical shelters, and empty canisters might also be moved around as decoys. The Soviets would therefore have to target warheads at each of the shelters to be certain of eliminating the missile; and Defense Department offi-



"We would fire it, Mr President, but we can't find it!"

cials claim that the cost of each warhead to the USSR would be significantly higher than the cost of each shelter to the US.

Members of Congress are keen to press ahead with the development of a mobile missile system, and are even suggesting that, since MX is unlikely to be deployed until the mid-1980s, a similar scheme known as alternative launch point (ALP) should in the meantime be developed for the existing Minuteman missiles. Given the increasing accuracy of Soviet missiles, the present Minuteman siloes distributed throughout the country will, it is claimed, soon be vulnerable to attack.

Others, however, are critical of the whole MAP scheme. "The notion that MAP provides an effective response to improved Soviet military technology falls into the fallacy of the last move, ignoring the likely response," according to Dr Michael Nacht, assistant director of the Program for Science and International Affairs at Harvard University. "If we build 20,000 holes for 1,000 missiles, and the Soviets then build 10,000 holes each capable of

holding a MIRVed (multiple inter-continental re-entry vehicle) missile with eight war-heads, how can we be sure that they do not cheat and confront us with 80,000 warheads?"

Further concern has been raised about the environmental impact of the scheme. A report published by the Air Force two weeks ago predicted that the total area required for full deployment of an MX force would be about 6,000 square miles, about the size of the State of Connecticut, and could be considerably more. In addition, the missile on its trailer would weigh over 500,000 pounds, and transporting the missile between different shelters would therefore make it necessary to strengthen large numbers of existing roads and bridges, currently designed to carry only 120,000 lb.

The Carter administration is now having to decide whether to press ahead with the next stage of the MAP scheme, a full-scale engineering development estimated to cost about \$5 billion over five years, or whether to concentrate its research efforts on strengthening the two other areas of missile delivery systems, the sea-land ballistic missile (SLBM) and long-range bombers.

The most immediate concern, however, is the impact of MAP schemes on the current SALT 2 talks. At present, proposals are being discussed for a three-year protocol agreed between the US and the USSR, which would accept mobile missile systems, but ban their deployment and flight testing from operational launchers.

At the same time President Carter has told the Soviets that, barring further agreement, the US reserves the right to deploy a mobile missile system in the post-protocol period—a position which the president feels necessary if a new SALT treaty lasting through to 1985 is to get the approval of Congress.

The Soviet response to this has been cool. While not ruling out the deployment of a mobile missile system under the new treaty, the chief Soviet arms negotiator, Vladimir S. Semyonov, has raised questions about the acceptability of the MAP system, in particular the difficulty that the USSR would have in verifying the total number of US missiles deployed at any one time.

"I don't think that a system of arms control based on verification is compatible with a system of missile deployment based on deception. And the implication is that either you have SALT, or you have MAP", according to Dr Nacht. Or perhaps you recruit your chess players as defence analysts, and give the World Championship a new meaning. □

Nuclear waste may be stored in synthetic rock

HIGH level nuclear waste may be locked into a synthetic mineral which is far more stable than the borosilicate glasses under development for the purpose in Europe and the US, an Australian geochemist said last week. Whereas glassified waste may devitrify when exposed to ground water at high temperature and pressure, thus exposing a large surface area for the dissolution of the radionuclides in the glass, the new mineral—'synroc'—should be as stable as a natural rock.

The scientist responsible, Professor A. E. Ringwood of the Australian National University, told *Nature* this week that "if people are prepared to pay the cost, they can get any degree of containment they require". The cost, Ringwood estimates, of the basic synroc process would be about 1% of the cost of the energy produced by the nuclear material from which the waste came.

"Our present system is just one example among many possibilities" said Ringwood. The objective was to pro-

duce crystalline minerals, related to known stable types, which would contain the dangerous radionuclides in a solid solution. The present synroc contains: perovskite (CaTiO_3), "very effective at dissolving rare earths and strontium"; a hollandite-like mineral ($\text{Ba}_2(\text{K})\text{AlTi}_2\text{O}_8$), capable of dissolving molybdenum, ruthenium, and rhodium; a cubic form of zirconia (ZrO_2), "a very refractory mineral which dissolves the long-lived actinides including uranium"; barium feldspar ($\text{BaAl}_2\text{Si}_2\text{O}_8$), which can immobilise barium, potassium, rubidium and caesium; and two others, calcsilite (KAlSiO_4) and lucite (KAlSi_2O_6) which can also dissolve caesium and rubidium. The mixture of minerals melts at $1,280^\circ\text{C}$ —low enough to use the same technology that produces borosilicate glass.

Ringwood's recipe is to melt 90% of the mixture with 10% of calcined high level waste, and recrystallise. It then forms a stable fine-grained rock "rather like a basalt". The waste is dis-

solved in the individual mineral crystals. "We are then proposing to bury the synroc 1 to 3 km down in impervious granite—one with few cracks" said Ringwood. Then little ground water will get to it; but any that does will find the mineral stable.

"We have demonstrated that the problem is soluble. But there can be variations". There may still be a problem in that there is an excess concentration of waste elements at the surfaces of the mineral crystals. But Ringwood believes he can deal with that: "leave the synroc for 20 years, crush it and wash in nitric acid to remove the surface-bound elements. Return the washings to the beginning of the whole process. Mix the remainder 1 to 4 with alumina and zirconia. Hot press the mixture into inert cylinders. Then no geological process could get it out." Cost of this fail-safe process? About 3% of energy costs.

Robert Walgate

Royal Society slams routine toxicity tests

MOST of the £50 million spent in the UK each year on the toxicity testing of new chemicals and drugs is wasted on "routine tests of limited value", says a Royal Society report published last week. These tests involve "the routine of exposure of many animals without attention to mechanisms. Only if mechanisms are understood is it possible to extrapolate toxicity measurements across species and from the large doses used in experimental animals to the exposures experienced by man, commonly a thousand-fold smaller".

More funds should be made available in the UK to study toxicology as an academic discipline and to identify people at risk from the effects of hitherto unknown toxic materials. These are the two main recommendations of the report which was produced by a Royal Society study group set up in 1975 under the Chairmanship of Sir Richard Doll to study long-term toxic effects.

As well as the mechanisms of toxicity other specific topics for further study recommended by the report include:

- the effect of nutritional and other variables on susceptibility to toxic effects;
- the carcinogenicity of chemical products. Current tests, says the report, are unlikely to provide a quantitative measure of carcinogenic risk to man.
- systems for monitoring trends in human disease. These are adequate for

providing data on mortality but give little information on the prevalence of non-lethal disease. The report recommends that the possibility of extending the work of the Office of Population Censuses and Surveys to include regular monitoring of ill-health should be considered.

The report also recommends that systems for monitoring the health of industrial workers exposed to new chemicals should be improved. Results of experiments on animals to test the toxicity of new materials cannot be extended to humans, it says, because of the lack of theoretical knowledge of the mechanisms of toxicity in different species. Hospital discharge and mortality data should also be linked to records of exposure to industrial and other hazards. □

Queue for life science in space

THE National Aeronautics and Space Administration has received an unexpectedly heavy response to its call for proposals for life sciences experiments to be carried out on Spacelab missions in 1981 and 1983.

"So far we have received over 340 proposals, and more are still coming in. This is an extremely large turn-out; in particular detailed proposals have been received from over 50% of those who originally submitted letters of intent,"

Dr David Winter, director of the life sciences programme at NASA, said last week.

Research areas from which the experiments will be selected include endocrinology and vestibular function, embryology, developmental biology and plant growth.

"The main attraction of Spacelab is the opportunity it provides for studying the role of gravity in life processes," according to Dr Winter. The presence of scientists will also allow "hands-on" experiments to be tried out, in contrast to previous life-science experiments in space which were fully automated.

"A number of the studies will be concerned with the behaviour of the human body in space. For example, there is a large redistribution of fluid and blood within the body in the first hours in space; whereas it would not be possible to carry out experiments at this early stage on crew members flying the vehicle, it will be possible to carry out studies on the experimenters themselves."

The potential contribution of such studies to later space shuttle flights was one of the factors which persuaded the Senate commerce committee to authorise an additional \$2 million for the administration's budget request of \$40.6 million in 1979 for life science experiments.

The high level of interest shown by life scientists is also being used by the agency to support attempts to increase the budget for life sciences in the 1980 budget.

David Dickson

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Camera Press

Smoke from a Czech factory pours into the air.

Friends of the Czech earth go underground

MOSCOW radio urged recently that environmental protection is an important field of international co-operation and detente, intimating, with quotes from Mr Brezhnev, that it is the West which is displaying "inconsistency and sluggishness" in this regard. Yet the first issue of a new Czech *samizdat* magazine, *Spektrum*, republished in London this week, includes a translation from *L'Express* (April 1977) of an extensive interview on ecological problems. If Czechoslovakia is following the Moscow line, why should such an article be *samizdat*?

At first glance there appears to be open discussion of problems of ecology and pollution in the Czech and Slovak press. Thus in April 1976, there was a whole spate of articles in the general and technical press on problems of atmospheric pollution, which in some cases actually presented concrete figures. *Zivotne Prostredie*, for example, gave the direct cost of air pollution as 4,400 million Kcs per year, and indirect damage (*ie*, damage solely attributable to corrosion) as an additional 2,000 to 2,500 million Kcs—figures which relate only to material damage, and take no account of health hazards.

Similarly, in 1977, the media devoted much attention to water pollution in Slovakia. According to Radio Bratislava (June 1977), in spite of numerous party and government decisions "outlining the way towards improvement", little had been done to construct the necessary recycling plants. A single issue of the weekly *Nove Slovo* (June 2, 1977) contained several articles on the problem, including a long interview with the Slovak Deputy Prime Minister Julius Hanus. He stressed that to deal with the 1,400 km of polluted water-courses in Slovakia, 500 new purification stations must be built, and that existing legisla-

tion on the use of recycling plants should be implemented more efficiently.

Discussion of environmental problems in the official Czech and Slovak media is thus nothing new. Why then did the 'publishers' of *Spektrum* find it necessary to circulate a western article on the subject.

Looking more closely at the contributions from the official media, it appears that such extensive discussions take place in conjunction with major party and governmental events. Thus the water-pollution "debate" was a preliminary to a special discussion of the problem by the Slovak National Council (and, as we have seen, included a trend-setting interview with Deputy Prime Minister Hanus).

Similarly, the publicity given to air pollution in April 1976 followed a speech by the party leader, Gustav Husak, at the 15th Congress of the Communist Party of Czechoslovakia, in which he urged that it was necessary "to make more determined efforts to reduce the harmful influences on the environment that ensue from the expansion of industry and transportation and the industrialisation of agriculture", noting that "it is our solemn duty to preserve the basic values of the environment, the wealth of nature, and the beauty of our country for future generations".

However, in spite of Mr Husak's stress on "environmental values", ecological planning, in Czechoslovakia as throughout the Comecon bloc, is closely linked to the basic concept of 'scientific and technical progress'. Industrialisation is a necessary good, a dogma incorporated in all planning without further discussion.

Any temporary deviations from this ideal seem purely pragmatic or political in origin. Thus in 1968, Czechoslovakia, which since the late 1940s had been exporting all her uranium ore to the Soviet Union, suddenly began to

stress the need to accelerate development of nuclear power. After the fall of Dubcek, however, this concept had to be abandoned for some years (with, incidentally, a resulting expansion in the mining of lignite—the chief cause of air pollution in Czechoslovakia). Now, Czechoslovakia plays an important role in the integrated Comecon plans for nuclear power: one major contribution by the Czech team being a detailed survey of the ecology of aquatic life in a radioactively-polluted water-course.

Against this background, the *samizdat* appearance of the interview from *L'Express* becomes meaningful. For the subject of the interview is Edward Goldsmith, a supporter of "green earth" type radical ecological programmes. His approach is not one of "doomwatch" statistics, although he does deal with various specific hazards, such as DDT.

Rather he is concerned with the philosophical and ethical problems underlying the concepts of ecology and conservation, strongly condemning 'materialism', whether of socialist or capitalist provenance.

It is not surprising that the Czech dissidents wish to discuss pollution: even the official literature admits the magnitude of the problem. In circulating the Goldsmith article they seem to be seeking a broader basis for discussion than that permitted by current Party doctrine.

Vera Rich

Erratum

In last week's issue, page 305, "The secret is to have no secrets", the first sentence should read, "Earlier this month, Anatolii Shcharanskii received a sentence of three years' imprisonment, plus ten years in a labour camp . . ."

Water holds Poland back, says Gierek

POLAND'S future "socio-economic development" may be "seriously hampered" by the shortage and inefficient use of water and the threat of its increasing pollution, according to a resolution of the Twelfth Plenary Session of the Central Committee of the Polish United Workers' Party which met earlier this month.

This resolution was a response to a massive report on the role of science in the country's development, presented by Edward Gierek, First Secretary of the Central Committee. Among many somewhat general statements—the need to save energy and natural resources, the growing importance of management efficiency, the expansion of research and the role of the Polish Academy of Sciences—the water problem was treated in far greater detail and in a tone of considerable urgency.

Until now, official Polish sources have seemed somewhat reluctant to allow water pollution to become a matter of public discussion. Enquiries from foreign specialists and journalists (including *Nature*) tend to meet with noncommittal statements implying that all is well, or at least well in hand. The evidence for pollution is therefore somewhat anecdotal—Sunday fishermen on the Vistula banks who stress that they would never dare eat their catch, or the Poznan joke about the man who fell into the city's artificial boating lake and was pulled out drunk on the waste waters of the brewery. Dissident sources would even claim that censorship regulations categorically forbid the reporting of certain aspects of water pollution at all.

Certainly the resolution of the Twelfth Plenum mentions pollution; but only, as it were, as a pending threat. The matters raised by Mr Gierek himself and for which the resolution proposes practical counter-measures are largely those of management.

Earlier this year, K. Frankowski, the Director of the Wroclaw provincial water conservancy, speaking on Warsaw television, pointed out that Polish water resources now come under no less than three ministries and numerous local authorities. In order to implement the existing "Outline of the long-term plan of water economy in Poland", he urged the establishment of a single responsible body to coordinate all measures dealing with water conservation.

Although Mr Gierek made no direct reference to Frankowski's proposals, he stressed that the water problem is "nationwide in scope" and must be "viewed, formulated and resolved" from this point of view. By the end of the century, he said, Poland will need an annual 40,000 million m³ of water, to satisfy the needs of the population, agriculture and industry. Poland's water resources are at the "lower end of the scale" for Europe. Nevertheless, he stressed, it is not their "global dimensions" which are the main problem, but their distribution.

Immediate proposals to improve the distribution of water resources are based on the development of the Vistula whose basin accounts for some 55% of Poland's surface water resources. Some 33% of Poland's urban population, and a significant proportion of industry are located in the five main conurbations on the river.

The development of the Vistula, said Mr Gierek, thus "occupies a key position in the whole of the water economy".

More practically, under the new scheme for the Vistula, some 220,000 ha of land, at present unused, would become available for intensive farming, and a further 1,000,000 ha arable land would be protected from flooding. The Vistula itself would become an up-to-date water-way, usable for an average of over 300 days a year, and removing the load from the other



Tranquil fishing in Poland, but the same anglers don't dare eat their catch.

sectors of the Polish transport system by some 100 million tonnes of cargo per year. Hydroelectric power stations at the locks could accommodate generating sets of some 16,000 MW and there would be considerable fringe benefits to town and country planning and to tourism.

On behalf of the Politburo, Mr Gierek proposed that the programme for the management of the Vistula and its water economy should be presented to the next Party Congress as "one of the most important tasks for the coming decades". Modestly, he suggested that should the members of the Central Committee share this opinion "it would be advisable" to express it in a separate resolution.

The resolution framed in answer to his appeal—which is some six times the length of that accepting the remainder of the science and technology report—essentially reiterates his main proposals, appeals to "scientists and all professions" and the whole nation to make a "creative contribution" to the development of the Vistula, and instructs the government to prepare a programme of works to be undertaken in 1981-85.

Vera Rich



The arrest of Podrabinek, April 1977.

Londoner may defend dissident

ALEKSANDR Podrabinek, the Moscow paramedic who wrote *Punitive Medicine*, a 265-page *samizdat* exposé of political misuse of psychiatry in the Soviet Union is expected to be brought to trial shortly under Article 191-1 of the Soviet Penal Code ("malicious slander").

Mr Louis Blom-Cooper, a London QC, who has been compiling a defence file on Podrabinek, has applied for a visa to attend the trial. Although this has not yet been granted, a fellow-lawyer, Mr Brian Wrobel, visited Moscow earlier this month and spoke

with representatives of various legal bodies in Moscow, none of whom ruled out the possibility of Mr Blom-Cooper's participation in the trial. One such person—the head of the Moscow city College of Advocates, has stated that if Mr Blom-Cooper telephones him before leaving London, he would be willing to see him.

Perhaps even more significantly, the Foreign and Commonwealth Office has backed Mr Blom-Cooper's visa request—an action which appears to be unprecedented, at least as far as the Soviet Union is concerned. □

correspondence

Temporary contracts

SIR,—In the editorial (29 June, page 265) you deplore the present legal restrictions on temporary research contracts as “another small restraint (which) has been imposed on freedom to do research”. The question is—freedom for whom?

I have been working for ten years as a (untenured) research fellow in the field of ultra cold neutrons. During this time I have achieved something of an international reputation and have made at least two contributions which have significantly altered the direction of research in this field. In spite of this I find that, due to the attitude of scientific administrators in this country to untenured researchers it is necessary for me to enter into collaboration with groups from other countries (in particular France, Germany and the USA) in order to see my ideas put into practice. In addition I must face the fact that my career as a researcher can be effectively terminated at the whim of a single professor who can simply refuse to apply for an extension to my research grant. There are at least three other people in this department in almost identical situations.

I think you and your readers should realise that the freedom of which you speak is bought at a terrible price, a price that is being paid by myself and my colleagues and I would ask you to be more tolerant towards a legal situation which offers us at least token support.

Yours faithfully,

ROBERT GOLUB

University of Sussex,
Brighton, UK

Burying high level wastes

SIR,—The reaction by Brookins *et al.* (29 June, page 704) to my suggestion (16 February, page 605) that conventional geochronological measurements might be more widely employed in the selection and evaluation of evaporite deposits for high-level radioactive waste disposal is based on a misinterpretation. It was certainly never stated either that “geochronologic K-Ar data argue against the suitability of bedded salt deposits” for this purpose or that all deposits, such as the New Mexico occurrence being considered as a waste repository, had been recently recrystallised by percolating groundwaters. On the contrary the limited results presented by these correspondents go some way towards illustrating the potential usefulness of isotopic techniques in evaporites’ evaluation.

That sylvite, a predominant mineral in some evaporites, loses radiogenic argon with the passage of time is “well-recognised” by the authors and is substantiated by their own results on the samples from Los Medanos and those of Polevaya *et al.* (*Geochem.* 8, 897–906, 1958). It is well-known that loss of a radiogenic isotope often occurs from the principal rock-forming mineral in some salt deposits. This surely is the all-

important point to be considered when discussing the suitability of these rocks for radioactive waste disposal.

The mechanism of loss is a secondary consideration. If diffusion is the favoured operative process, as has been implied, it is surprising that identical coegenetic crystals from the same deposit have different argon contents at the present time (as appears to be the case with the sylvites from the Los Medanos deposit, for example). Other loss promoting processes, such as recrystallisation (Polevaya *et al.*) require consideration and it would therefore be of considerable interest to examine their bulk distribution and timing through a systematic programme of argon measurements.

That the rare mineral langbeinite is highly retentive of radiogenic argon has been known for over a decade (Lippolt, H. J. and Oesterle, F. P. (*Naturwiss.* 64, 90; 1977 and references therein) so it comes as no surprise that the ‘correct’ age was obtained for the single Los Medanos sample. Unfortunately this is of little consequence as no proposals have been advanced to dispose of radioactive waste in monomineralic deposits of this type.

In summary it is misleading of Brookins *et al.* to blandly dismiss the K-Ar evidence and attribute the results solely to a selective diffusion process (which operated exclusively on gas atoms in sylvites). This attitude only encourages others (eg Cohen, *Rev. Mod. Phys.* 49, 10, 1977) to state that (for salt beds) “their very existence over time spans like 250 Myr is proof that water has not entered them”. Such pronouncements require scientific justification and I reiterate my point that detailed geochronological investigations would appear capable of assisting in the location of those potential salt repositories offering maximum security.

Yours faithfully,

R. M. MACINTYRE

University of Strathclyde, UK

Saccharin

SIR,—B. L. Cohen makes an erroneous assumption in his letter on saccharin (29 June, page 704) when he assumes that “it would be highly beneficial to the public if the food processing industry were to substitute saccharin for sugar”. His assumption is that the consumption of food calories would diminish by an amount equal to the sugar calories of sweetened foods, if saccharin were substituted for sugar. What would happen, of course is that the same number of calories would be consumed in a different form, by eating the same weight of food, because food is eaten to supply calories. If the article of food were high in fat, the hazard to health might be increased.

Yours faithfully,

THOMAS H. JUKES

University of California,
Berkeley, USA

Human rights world-wide

SIR,—As scientists concerned about human rights, we are disturbed by the recent boycott by United States’ physicists of conferences in the Soviet Union. Their action reflects the current political climate that stems largely from the Carter administration’s policy on human rights. However, while the “Human Rights Campaign” vociferously condemns trials of dissidents in the Soviet Union, it ignores or minimises flagrant violations of human rights in South Korea, Iran, and other allied countries for reasons of “national security”. This campaign is equally inconsistent in its steadfast refusal to recognise violations of human rights in the United States.

We believe it is our duty first to protest human-rights violations in our own country. The case of the Wilmington ten has been cited by Amnesty International (recipient of a Nobel Peace Prize) as a violation of human rights. Ten civil rights workers, eight of them high school students, demanding equal education in Wilmington, North Carolina, defended a church from armed attack by the Ku Klux Klan in 1971 and subsequently were framed on an arson charge. The Wilmington ten are serving prison sentences averaging 28 years and have been political prisoners now for more than two years. Seventy-five Congressmen and women have asked US Attorney General Griffin Bell to intervene. Church, community, labour, and political leaders in this country and abroad are demanding their release. Nor is this an isolated case. The human and constitutional rights of native American Indians, Spanish-speaking Americans, black Americans, women, and members of specific political groups are infringed daily.

Is it not hypocritical for Americans to condemn the Soviet Union when our own country is culpable? Certainly criticism of other nations would be more convincing were the domestic record not so flawed.

We are dismayed that American physicists have chosen to follow our government’s policy of directing attention away from injustices in the United States by trying to focus it abroad. Such actions are even more regrettable in view of the prolonged and extensive efforts by American scientists to establish programmes of cooperation and exchange with their Soviet colleagues. Rather than a demonstration of support for worldwide human rights, the boycott abets the masking of violations.

Yours faithfully,

ROY S. CHALEFF

HENRY S. LOWENDORF

Cornell University,
New York, USA

news and views

Myosin: polymorphism and promiscuity

from Alan Weeds

SKELETAL muscles may be classified broadly into two types according to their function. Those involved in maintaining posture are called 'slow' because they contract relatively slowly with a sustained pattern of activity, while 'fast' muscles shorten more rapidly and are used for intermittent bursts of more vigorous movement. Differences between these two types have been demonstrated by a variety of physiological, histochemical and biochemical techniques. They have different frequencies of innervation by their motor neurones and contain different myosin isoenzymes. Indeed it has become an axiom that the maximum speed of shortening of a muscle is controlled by the rate of turnover of ATP by its constituent myosin isoenzyme (Barany *J. gen. Physiol.* **50**, 197; 1967).

What factors regulate the myosin genes that are expressed in a particular muscle? Following the pioneering work of Buller, Eccles and Eccles (*J. Physiol. Lond.* **150**, 417; 1960), it has become clear that the pattern of nerve stimuli reaching a muscle determines its characteristic response. Thus cross reinnervation, whereby a nerve originally connected to a slow muscle is cut and reconnected to a fast muscle and *vice versa*, produces a reciprocal transformation of the muscles' properties including the type of myosin isoenzyme synthesised (Buller *et al. J. Physiol. Lond.* **205**, 581; 1969; Weeds *et al. Nature* **247**, 135; 1974). Similar changes have been observed by using long term electrical stimulation of fast muscles at a continuous low frequency to mimic slow muscle innervation: myosin characteristic of slow muscle is produced and the muscle is completely transformed to a slow type (Salmons & Sreter *Nature* **263**, 30; 1976). Current evidence suggests that no new muscle fibres develop, but that transformation is produced by a change in the programme of protein synthesis

within pre-existing fibres (Rubinstein *et al. Fedn Proc.* **36**, 584; 1977; Pette & Schnez *FEBS Lett.* **83**, 128; 1977). Both types of experiment indicate that gene expression is regulated by the nervous system.

Many muscles do not contain a single population of either 'fast' or 'slow' fibres, but are composed of a mosaic of different fibre types, which can be identified by histochemical staining or reaction with specific myosin antibodies (Gauthier & Lowey *J. Cell. Biol.* **74**, 760; 1977). Each class of fibres is innervated by its own specific motor neurones. Such a mixing of fibre types produces a great variety of contractile responses to accommodate individual muscles to their particular functions. Within a single fibre type it has previously been assumed that only one class of myosin isoenzymes will be present under normal conditions (that is, where the fibre population is not undergoing transformation due to surgical or other artificial manipulation). As evidence for this, polyacrylamide gel electrophoresis shows distinct differences in the mobilities of native myosins isolated from fast and slow muscles (Hoh *et al. Biochem. J.* **157**, 87; 1975). But recent experiments on developing muscles have cast considerable doubt on the validity of this assumption. Since myosin is synthesised and assembled into myofibrils long before muscles are innervated, we would like to know which myosin genes are expressed in these early myogenic cells. Is only one of the polymorphic classes of myosin synthesised or are the cells promiscuous in producing both classes initially and only after innervation do they differentiate into specialised fibres of one particular type?

Attempts to identify the particular isoenzymes present in developing muscles have relied on the characteristic pattern of myosin light chains obtained on polyacrylamide gel electrophoresis in sodium dodecyl sulphate, or on the use of specific myosin antibodies. According to these criteria, embryonic chick pectoralis muscle (a fast muscle in the adult) contains only fast type

myosin, while embryonic anterior latissimus dorsi (a slow muscle in the adult) contains both types and there is a progressive decrease in the proportion of fast myosin during its development (Rubinstein *et al. Proc. natn. Acad. Sci. U.S.A.* **74**, 4524; 1977). The authors suggest that myosin similar to adult fast type is synthesised initially in both presumptive fast and slow muscles and only at a later stage, probably as a result of intervention, is slow myosin produced. Similar conclusions have been derived from studies of muscle development in postnatal rats (Rubinstein & Kelly *Devl Biol.* **62**, 473; 1978).

A slightly different approach was used by Gauthier *et al.* (*Nature* **274**, 25; 1978) to identify the myosin types present in fibres of rat diaphragm muscle during early development. Cryostat sections of muscle were exposed to purified myosin antibodies and the complexes visualised by immunofluorescence. Although in the adult rat the diaphragm muscle contains a mosaic of different fibre types, at 19 days gestation all the fibres stained with antibodies to both fast and slow myosins. Even up to the third week of postnatal development all fibres showed the presence of both myosins but differential responses became apparent as development proceeded. These results suggest that all fibres in developing rat diaphragm muscle synthesise both classes of myosin simultaneously. This might be explained by multiple innervation, as it is known that each fibre receives axons from two or more neurones during the early phases of innervation. Such an explanation could also account for the widely held view that embryonic muscles are characteristically of the slow type. But the observation is not restricted to rat diaphragm muscle, since Masaki and Yoshizaki (*J. Biochem.* **76**, 123; 1974) showed the presence of fast and slow myosins by fluorescent antibody staining in embryonic chicken breast muscle. Thus both of these results are in conflict with those reported by Rubinstein and his collaborators.

Alan Weeds is on the Scientific Staff of the Medical Research Council at the Laboratory of Molecular Biology, Cambridge.

Part of the discrepancy between these findings may be due to the techniques used and they illustrate the caution needed in interpreting immunochemical experiments. Immunofluorescence is highly sensitive and detects both soluble and precipitable complexes, but its reliability depends crucially on the purity of the antibodies used. Gauthier *et al.* used highly purified myosin as an antigen and purified the antibody preparations by immunoadsorption and affinity chromatography. Rubinstein *et al.* also used specific antibodies but relied on immunoprecipitation to identify the complexes, and this procedure would fail to detect non-precipitable or low affinity reactions. Rubinstein *et al.* also used polyacrylamide gel electrophoresis in sodium dodecyl sulphate to identify the myosin isoenzymes, but this method may not be sufficiently sensitive, as Rubinstein and Kelly tacitly acknowledge in a footnote to their paper. Recently evidence has been presented for an embryonic form of myosin, which has been characterised by its light chain mobility on 2-dimensional gel electrophoresis (Whalen, Butler-Browne and Gros, private communication) and this light chain could not be distinguished from that of fast

muscle myosin by electrophoresis in sodium dodecyl sulphate. Whether this myosin cross-reacts with antibodies against fast or slow myosins is not yet known. But the possible presence of an embryonic form of myosin adds further complexity to the problems of understanding muscle development.

At present it is not possible to state whether a single form of myosin is endogenously programmed in early myogenesis, although studies of myosin synthesised in tissue culture preparations seem to favour this hypothesis. Polyneuronal innervation may account for the promiscuous behaviour of certain developing muscle cells, but much more evidence is needed to establish this. Certainly there is now good evidence from all these studies for the coexistence of different myosin types within single fibres both in developing muscles and in Purkinje cells from chicken hearts (Sartore *et al.* *Nature* **274**, 82; 1978). The number of polymorphic forms of myosin identified in different muscles increases steadily as techniques become more sensitive. These isoenzymes should provide useful markers to unravel the early events in myogenesis and the differentiation of muscle fibres following innervation. $\square$

How many quarks?

from F. E. Close

In November 1974 the discovery of the J/Ψ particle in the debris of proton-proton collisions and also in electron-positron annihilations signalled the closing of a chapter in high energy physics. The J/Ψ properties and those of similar particles subsequently found in electron-positron annihilation showed that a new quantum degree of freedom (charm) exists in Nature. This implied that the three known flavours of quark (up, down and strange) were accompanied by a fourth—the charmed quark. This yielded a pleasing symmetry between the world of four leptons (electron, muon and two neutrinos) and that of the quarks.

The familiar world is built from the electron and its neutrino in the lepton world and the up and down quarks. These are referred to as the 'first generation of elementary fermions' (*News and Views* **271**, 406; 1978). It is probable that such a symmetry between the leptons and quarks is fundamental and necessary if the weak and electromagnetic interactions are to be successfully unified. Indeed it was in the attempt to formulate such a unification that the charmed flavour of quark had earlier been predicted in order that a second generation of fermions be com-

pleted (muon and its neutrino, strange and charmed quark).

The discovery of the J/Ψ and charm would have elegantly sealed things with two generations of fermions had it not been for the unexpected discovery in 1975 of a heavy lepton, τ . This lepton's existence has been recently confirmed by data from Hamburg (the electron positron colliding beam facility DORIS) (*News and Views op. cit.*). There is some indirect evidence that this τ lepton also has a brother neutrino. Hence a third generation of lepton seems to exist.

To maintain the quark-lepton symmetry theorists speculated that two new quarks should exist (named top and bottom) with charges $2/3$ and $-1/3$ of a proton's charge.

If particles containing top or bottom quarks exist, they must be much more massive than anything previously found as no evidence for them has ever been found at low energies. Just as the J/Ψ is a bound state of $c\bar{c}$ (charmed quark and its antiquark), so an analogous massive T_b and T_t should exist made from $b\bar{b}$ and $t\bar{t}$ respectively. These T should be produced in the debris of proton-proton collisions and in electron-positron annihilation analogous to the Ψ discoveries.

The first evidence for an Y emerged in the summer of 1977. At Fermilab near Chicago a massive Y (three times as massive as Ψ and ten times the proton) was seen in muon pairs produced by proton-proton collisions. This has been confirmed by work at the CERN-ISR in Geneva, and an Y' some 600 MeV more massive than Y has also been found. This is tantalisingly the same mass separation as $\Psi' - \Psi$ and so theorists speculate that these Y are bound states of a new heavy quark in a logarithmically rising potential (since this leads to splittings in mass for $\Psi' - \Psi$ and $Y' - Y$ independent of the mass scale) (*News and Views op. cit.*).

Some properties of the production suggested that it was the $Y(bb)$ that had been found. This could be best tested by observing the Y in electron-positron annihilations. The coupling strength (technically the leptonic width) is proportional, in particular, to the square of the charge of the b (or t) quark. If it is $Y(bb)$, then the leptonic width would be about 1 keV whereas for $Y(t\bar{t})$ it would be similar to that of the Ψ (the t and c have the same charge) and hence about 4 keV.

No observation of the Y had been made in electron-positron annihilation as the highest energies attainable at SPEAR (California) and DORIS (Hamburg) were slightly too small to produce it. In a year or so, higher energies will be available when DORIS is replaced by PETRA at Hamburg. In DORIS' final days an attempt was made to increase its energy slightly to see if the Y could be detected.

Two groups (DASP and PLUTO collaborations) (*News and Views* **273**, 705; 1978) have detected a metastable resonance with mass of 9.46 GeV and both are agreed that the leptonic width is 1.3 ± 0.4 keV. This points very clearly towards the Y containing a bottom quark.

How many Y ?

Many particles like the Ψ have been found in the debris produced in electron-positron annihilations. These particles are bound states of a charmed quark and charmed antiquark and their properties are consistent with their being the two $1S$, three $1P$, and $1D$ and a $2S$ level of a charmonium spectroscopy (analogous to the positronium spectroscopy of atomic physics). These states are all metastable and easily studied as a result of them being below the threshold for pair production of charmed particles. Heavier charmonium states are less easily isolated as they are very short lived, decaying rapidly into pairs of charmed particles.

It has been noted by several authors that if non-relativistic potential pictures are applicable, then if bound states of more massive quarks exist, the number of metastable S states producible directly in e^-e^+ annihilation will grow as the

quark mass scale grows. For the Υ family it is predicted that 1S, 2S and 3S levels will be metastable. The 1S and 2S exist around 9.4 and 10.0 GeV mass. It is hoped that DORIS may be able to reach up to 10.5 GeV or so to see if a metastable 3S state also exists. If so, then an unprecedented laboratory will have been found for studying quark dynamics. Similarities and differences with the Ψ family can be studied and the role of the quark mass disentangled.

Guaranteed discoveries

We can look forward to the crossing of the threshold for pair production of 'naked bottom' particles (analogues of charmed particles), and the isolation of these particles. Given that the Υ is metastable there is much to be learned by studying its decay systematics and comparing with those of the Ψ . The theory of strong interactions recently developed (quantum chromodynamics) makes some specific predictions here which can be readily tested.

All this promises much exciting work to be done as soon as PETRA begins operation at higher energies than have ever before been attained. The real challenge, now brought into sharp focus, is to find evidence for the top quark and so to complete the third generation of quarks. If the $\Upsilon(t\bar{t})$ is found at 15 to 20 GeV say, then 1S through 5S levels of the $t\bar{t}$ spectroscopy could all be metastable! The information that these could yield about quark dynamics would be significant indeed.

The art of good theatre is to make a dramatic exit. The DORIS facility is certainly doing that. In turn, PETRA which supersedes DORIS, has been opened. The discoveries that are almost 'guaranteed' here are very exciting to anticipate and will generate several years of intense activity.

And when this is all done, will three generations of fermions be the end of the road? Or will another unsuspected lepton turn up and open another Pandora's box of quark flavours and metastable particles? Watch this space. □

A black hole in the centre of M89?

from James Pringle

THE nuclei of galaxies are extraordinary regions of the Universe. A typical galaxy consists of several hundreds of billions of stars spread over a region of space some 50 kiloparsecs across. Thus the average density of stars in a galaxy is about 10^{-3} per cubic parsec. The stars are bound together by their own self-gravity orbiting at speeds of a few hundred kilometres per second and with periods of the order of a hundred million years. Yet despite the overall sparseness and slowness of a galaxy as a whole, the galactic nucleus—the small central region less than a few hundred parsecs across, with a stellar density often exceeding several thousands of stars per cubic parsec—can display violent activity on time-scales of less than a week and emit energy and radiation at a rate that exceeds the combined luminosities of the stars comprising the galaxy as a whole.

An extreme example of this phenomenon is thought to occur in QSOs which appear to be elliptical galaxies in which the emission from the galactic nucleus outshines the rest of the galaxy by a factor that can exceed a thousand. This enables these

objects which are comparatively rare in the Universe to be seen out to vast distances, or, equivalently, at large redshifts. More indirect evidence of the power that can be produced by a galactic nucleus is given by the existence of double radio sources on either side of a central galaxy. A recent paper (Readhead *et al.* *Nature* **272**, 131; 1978) reported evidence of a narrow jet in the elliptical galaxy NGC 6251 that could be traced in all the way from the outer radio lobes, at a distance of over a megaparsec from the central galaxy, to within 2 parsecs of the central nuclear radio source. It has long been known that the energy needed to power some of the larger radio double sources through their lifetimes is equivalent to the rest energy of tens of millions of solar masses. The evidence strongly suggests that all this energy was produced in the nucleus of the central galaxy.

One of the most efficient means known of converting matter into radiation is by accretion onto a black hole. A conversion efficiency exceeding 10% is expected theoretically: this compares favourably with nuclear burning for which the conversion efficiency is less than 1%. It is not, therefore, surprising that the most luminous objects in the Universe are thought to be powered by accreting black holes. Moreover, it has been argued, in

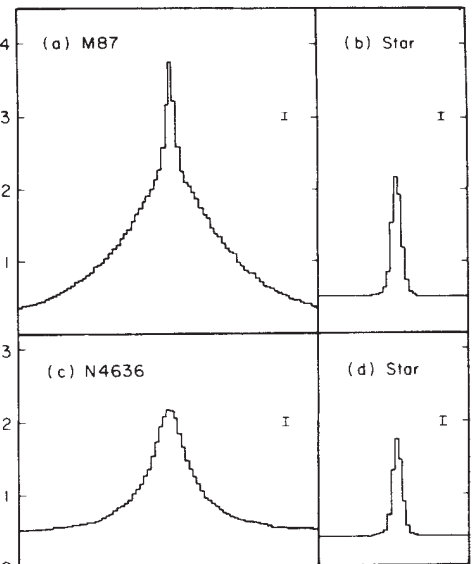


Fig. 1 V band photometric profiles depicting a 1×80 pixel slice from the SIT data for *a*, M87, showing the abnormal central luminosity spike; *b*, a star profile for the M87 data with $\sigma = 0.67''$; *c*, NGC 4636, a typical elliptical galaxy profile; *d*, star profile for NGC 4636. Each pixel is $0.5''$ wide and the intensity scale is linear. Error bars of length 2σ are given.

particular by Martin Rees (see *Nature* **265**, 402; 1977), that essentially all non-black hole schemes that have been thought up to explain the high luminosities required in QSOs have, as the natural end point of their evolution, the formation of a massive black hole. The question then arises as to whether the existence of a massive black hole may be a widespread phenomenon, with massive black holes lurking in the nuclei of galaxies which are at present relatively quiescent. If the black hole is large enough, it should be detectable by its influence on the orbits of the stars in the region of the nucleus.

As mentioned above, the central region of a galaxy consists of a dense cluster of stars. The density profile of such a self-gravitating star cluster should be smooth in the centre. In other words, if the apparent surface brightness of the cluster is plotted as a function of distance from the cluster centre, the graph should appear, like a Gaussian distribution, smooth and round-topped. On the other hand, if such a star cluster contains a non-luminous point mass, the density of stars in the neighbourhood of the mass increases in such a way that the surface brightness plot is distorted to appear cusped in the centre. The larger the point mass, the further out in the cluster is the distortion apparent. Moreover, although in the centre, or core, of the star cluster the dispersion velocity of the stars, measured by the breadth of the lines

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in the spectrum, should be roughly constant, in the neighbourhood of such a central point mass, the stellar dispersion velocity should show a dramatic increase.

Evidence for both these phenomena, the cusp in the brightness distribution and the increase of central dispersion velocity, have been reported by Young *et al.* (*Astrophys. J.* **221**, 721; 1978) and by Sargent *et al.* (*Astrophys. J.* **221**, 731; 1978) in observations of the nucleus of the galaxy M87. The peculiar EO galaxy M87 (EO implies that it is an elliptical galaxy which appears spherically symmetric on the sky) was chosen as a promising candidate in which to search for a central massive black hole for a number of reasons. It is relatively close to us for its size, being some 15 Mpc distant. Its nucleus is known to contain an active, compact radio source (less than 0.1 parsec in size) and jets are seen emanating from the centre of the galaxy at radio and optical wavelengths.

Some of the brightness distribution data from the paper by Young *et al.* is shown in Fig. 1. It is immediately evident that there is a sharp peak in the central region of M87 whereas, in comparison, the brightness distribution of the central region of the elliptical galaxy NGC 4036, which displays a profile typical for elliptical galaxies, appears rounded and smooth. Further investigation shows that the peak is too broad to be due just to an additional central point source of radiation but is consistent with an increase of stellar density within the innermost 400 parsecs. The spectroscopic observations of Sargent *et al.* show that the velocity dispersion rises in the centre reaching a maximum observed value of 350 km s⁻¹ at a distance of 100 parsecs from the centre.

Although the authors are careful to point out that these data do not prove the existence of a central, massive black hole, the data do demand an anomalous central mass of about 5×10^6 solar masses within the innermost hundred parsecs. The mass to luminosity ratio of the disturbing agent must exceed that of the observable stars by at least an order of magnitude. Although alternative explanations do exist (for example the mass could consist of a dense cluster of some tens of billions of compact stellar objects such as neutron stars or white dwarfs), the authors conclude that the existence of a central black hole is the most plausible explanation of the data at hand, and that, at present, M87 is the best case for the existence of a massive black hole in a galactic nucleus. They also note that the order of magnitude increase in resolution that will be provided by the

Space Telescope will not only make feasible a more detailed study of M87 but will also raise the possibility of searching for similar phenomena in more distant elliptical radio galaxies.

X-ray sequencing of proteins by refinement

from C. C. F. Blake

WHEN high resolution electron-density maps of proteins are produced by X-ray diffraction analysis of crystals, they are normally interpreted with the aid of an independently determined amino acid sequence. From time to time, however, maps are produced of proteins for which there is no current sequence information. This has provided the incentive for various attempts to produce the sequence directly from the electron-density maps by identifying the individual amino acids along the polypeptide chain. This procedure has some serious problems that can be considered under three heads.

First, it must be realised that the identification of a particular amino-acid residue is made almost entirely in terms of its shape. This leads immediately to a source of ambiguity, because certain pairs of amino acids are isostructural: Asp and Asn; Glu and Gln; and Val and Thr. Other pairs although not truly isostructural are sufficiently alike in shape for confusion to arise in all but the very best maps: Asp or Asn and Leu for example, differ only in shape at the γ -fork which is planar in one case and pyramidal in the other, while the five-membered imidazole ring of His may not always be readily distinguishable from the benzene ring of Phe. Of course environmental effects such as the location of the residue in the interior or the surface of the protein, or the presence of groups within hydrogen-bonding distance or not can help, but may not always prove decisive.

Second, the appearance of a particular type of residue will be critically dependent on its location in the protein molecule. In a fairly rigid environment, say in the protein core, its shape may be well-defined, but located in a more flexible region, say on the protein surface, will tend to broaden the electron density envelope, perhaps differentially so, giving it a

vague or even misleading shape. In the limit the electron density can broaden to invisibility, particularly for long polar side chains, making their identification almost impossible.

The two sorts of difficulties outlined above would occur even in perfect electron-density maps, except that at resolutions better than 2 Å the difference in electron-density between carbon and oxygen atoms should become noticeable, allowing the Val-Thr ambiguity to be resolved. In actual electron density maps experimental error will of course have an effect, and it will tend to blur and distort the envelopes of the side chains making identification even more problematic than it is in the ideal case. All these problems were discovered and discussed in the earlier attempts to derive sequence from Fourier maps, starting with myoglobin (Kendrew *et al.* *Nature* **190**, 666; 1961) and continuing with carboxypeptidase (Lipscomb *et al.* (*Proc. natn. Acad. Sci. U.S.A.* **64**, 28; 1969), thermolysin (Colman *et al.* *J. molec. Biol.* **70**, 701; 1972) and rubredoxin (Herriott *et al.* *J. molec. Biol.* **80**, 423; 1973).

Although for all these proteins, high-quality electron-density maps were available at resolutions close to 2 Å, comparison of the 'X-ray sequence' with the chemically derived sequence showed that even for the tiny rubredoxin molecule the success rate was only 74%, while for the other three much more typical proteins it was no better than 60%. What then can be done with a significantly larger protein at a lower resolution than any of these four? This is the question asked, and answered, by Steitz and his colleagues in two recent papers (Anderson *et al.* *J. Molec. Biol.* **123**, 1, 15; 1978). Working on yeast hexokinase, whose polypeptide chain is about 460 residues long, they had an isomorphously phased map at 2.5 Å, but no independent sequence information. Since the success rate of the X-ray sequence of rubredoxin had been increased to 93% by crystallographic refinement at 1.5 Å, they decided to examine the value of this technique on a protein nearly 10 times bigger with a lower resolution (2.1 Å).

Crystallographic refinement provides an answer to the important question: what is the difference between a proposed model of a molecule and 'reality'? Provided that the model is reasonably close to reality—and this can be achieved by interpreting an isomorphous map in terms of main-chain conformation and a proportion of the side chains—then the differences between it and the actual molecule can be seen in a difference electron-density map. The technique is particularly appropriate to amino acid identification

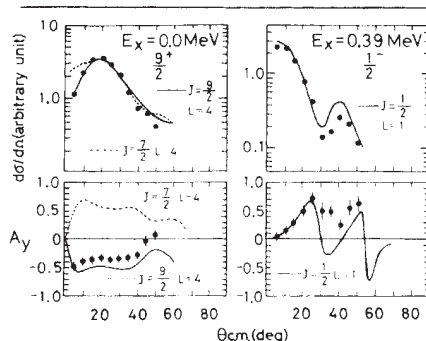
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NUCLEON transfer reactions, in which a nucleon or group of nucleons is transferred from one nucleus to another, are already established as one of the most powerful ways of determining the properties of nuclear states. The angular distribution is usually sensitive to the transferred angular momentum L and this provides important information about the final state of the residual nucleus. The analysis is particularly simple if the target nucleus has no regular momentum, for then the angular momentum J of the final state is $L \pm \frac{1}{2}$, where L is the transferred orbital angular momentum and the $\frac{1}{2}$ is the spin angular momentum. If L is determined from the angular distribution there still remains the problem of deciding between $L + \frac{1}{2}$ and $L - \frac{1}{2}$. This can be done from theoretical considerations, but it is still desirable to have an experimental method of resolving this ambiguity.

This is provided by the measurement of the polarisation of the emitted particles or by a rather similar quantity, the asymmetry of the particles from a reaction initiated by polarised projectiles. The usefulness of this method has recently been studied for the (p, α) reaction with polarised protons by Mikumo and his colleagues at the University of Tsukuba in Japan (*Phys. Rev. Lett.* **41**, 16; 1978).

Nuclear spectroscopy with (p, α) reactions

from P. E. Hodgson



Differential cross sections and asymmetries of the $^{118}\text{Sn}(p, \alpha)^{113}\text{In}$ reaction to the ground and 0.39 MeV states of the final nucleus, compared with distorted wave calculations with various values of J and L .

They irradiated ^{118}Sn and ^{112}Cd with 22 MeV polarised protons and measured the cross sections and asymmetries of the reactions leaving the residual nuclei in a number of different final states. Some of the results are shown in the figure, in which the cross sections and asymmetries are compared with distorted

wave calculations for various values of J and L . In the case of the reaction to the ground state of ^{113}In , the calculated differential cross sections for $J=9/2$ and $J=7/2$ are very similar, but the asymmetries are quite different, so that it is possible to conclude that the state has $J=9/2$ without any ambiguity. In the same way the state at 0.39 MeV is found to have $J=\frac{1}{2}$.

The distorted wave calculations are made with the cluster approximation in which the three transferred nucleons are considered as a triton cluster, with the appropriate wavefunction. This is rather a drastic approximation, and the generally good agreement between theory and experiment is a confirmation of its validity for the (p, α) reaction. This is important for the use of the reaction in nuclear spectroscopy because if this approximation is not made it is necessary to consider the three transferred nucleons individually, and this makes the calculations impractically complicated.

This new work shows that the analysing power of the (p, α) reaction is a sensitive way of determining the angular momenta of nuclear states, and the comparative simplicity of the analysis should may it widely useful.

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because it should indicate clearly where too few or too many atoms have been assigned to a particular side chain. For example, if, when interpreting the isomorphous map, a tyrosine is mistaken for a phenylalanine then the difference map should indicate that an additional atom—the oxygen of the terminal hydroxyl group—should be included (Steitz shows a nice example of just this effect). The process is comprehensive and iterative, so that the inclusion of the newly discovered hydroxyl group in the next difference map will improve features indicating the inclusion or exclusion of atoms in other residues, which in turn will improve the subsequent map, and so on. Effectively this enables errors due to poor isomorphous phasing to be progressively eliminated, and also enables the resolution to be increased without using heavy atom derivatives.

Clearly exhaustive use of refinement can result in a reasonably close approximation to the true electron density distribution, which means that the limitation to determining the amino acid sequence in this way is the number of residues that are intrinsically not well-ordered in the protein. Although Steitz has improved the

amino acid identification from no better than 25% in the isomorphous map to 69–70% at the end of the refinement, it is evident that there are fewer Glx and Lys residues, and more Ala and Ser, than there should be. This is a consequence of the disordering of the longer residues and their inevitable misidentification as smaller residues. It is difficult to see how any conceivable X-ray method of sequencing can deal with the problem of mobile surface residues, and it would seem to present a fundamental limitation to the technique.

Nevertheless identification of 60–70% of the amino acids in a protein can be valuable on at least two counts. First the knowledge of the nature of particular side chains involved in substrate binding or catalysis in the active site is clearly important. Secondly, the identification of a proportion of the amino acids will facilitate the fitting of peptides, even very short ones, when they become available at the beginning of chemical sequence analysis, and should enable peptides to be correctly assembled in an end-to-end manner, making unnecessary the overlapping peptides normally used for ordering. □

Renewing the search for calcium pumps

from V. L. Lew

It is now generally accepted that the maintenance of the large inward Ca^{2+} gradient across the plasma membranes of living cells is an essential condition for cell survival and for the various physiological roles of calcium as a trigger, signal-effect coupling agent or second messenger. What is not so clear is how these gradients are maintained.

The view most generally held at present is that in excitable cells and in many non-excitable cells as well, Ca extrusion is mediated by a $\text{Na}:\text{Ca}$ countertransport mechanism which derives its energy from the electrochemical gradient of Na and perhaps also K . In red cells and certain cultured cells on the other hand an ATP-fuelled Ca pump is responsible for the uphill extrusion of Ca . Although the possibility has been raised that these apparently separate transport mechanisms reflect different operating

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modalities of the same molecular apparatus, this is not very likely since all efforts to select alternative translocation modes of the red cell Ca pump have so far failed.

Over the past few years however, evidence that ATP is involved in the extrusion of Ca in squid axons has been interpreted as indicating interaction between ATP and the Na:Ca countertransport mechanism. An interesting alternative is that Ca extrusion is in fact mediated by red-cell-type pumps in most if not all, cells, and that the Na:Ca countertransport, when present, operates in parallel with this pump, either as an extra Na or extra Ca 'pump' depending on the relative electrochemical gradients of these two ions. (Mullins in *Membrane Transport Processes* 2, 371 (eds Tosteson, Ovchinnikov & Latorre) (Raven Press, 1978)). Some support for this view has been provided by the elegant experiments of DiPolo (*Nature* 274, 390; 1978) who has shown that in the absence of Na gradients there is an ATP-dependent uphill extrusion of Ca across the squid-axon membrane, a convenient tissue for research on the Na:Ca countertransport.

But the real distribution of plasma-membrane Ca pumps has yet to be determined. However, recent findings concerning the red cell (Mg+Ca) ATPase can be applied to the reinvestigation of Ca pump distribution.

Although (Ca+Mg) - stimulated ATPase activity has been reported in microsomal preparations of many origins, what makes the widespread existence of red-cell-type Ca pumps so uncertain is the lack of specific Ca pump inhibitors; the highly compartmentalised distribution of Ca in nucleated cells which complicates the interpretation of Ca-flux data in intact cells; and the bewildering variability of kinetic parameters of the Ca-stimulated microsomal ATPases which contrasts with the invariance exhibited by other ATPases associated with ionic pumps like the Na pump or the sarcoplasmic reticulum Ca pump.

Similar variability is also exhibited by the red cell Ca pump. Minor differences in the treatment of intact cells and in the preparation of the resealed ghosts or membrane fragments, can produce changes in the apparent K_m for Ca of nearly three orders of magnitude (from, say, 1 μ M to 1 mM) as well as up to 40-fold differences in V_{max} . Similar inconsistency among enzymes of different origin is hardly proof of identity but if we understood the reasons for the variability of the red cell (Ca+Mg) ATPase we might learn how to obtain consistent behaviour and try the trick on the other

enzymes.

Recent experiments with the red cell (Ca+Mg)ATPase suggest two main reasons for the observed variability. In 'broken' membrane preparations, the ATPase activity is, at least in part, associated with vesicular membrane fragments. Although these vesicles must be substantially more permeable to Ca than the original membrane, the maximum rate of Ca extrusion through the pump is so high that it can easily exceed the rate at which Ca can be supplied to the pump from outside the vesicles. Under these conditions Ferreira (thesis, Cambridge University, 1977) has pointed out that the actual Ca concentration inside the population of vesicles with normal topology might be considerably lower than that in the bulk medium. The apparent Ca affinity would therefore look lower than the true Ca affinity of the pump and the kinetics of Ca activation might also be considerably distorted depending on the proportion of membrane area forming right-side out vesicles. The ionophore A23187 is known to incorporate readily into red cell membranes increasing their effective Ca permeability. When this ionophore was added to red cell (Ca+Mg)ATPase preparations having a low apparent Ca affinity, Scharff and Foder (*Biochim. biophys. Acta* 483, 416; 1977) found that the high Ca affinity believed to represent the true affinity of the undisturbed enzyme was often, but not always, fully recovered. Vesicle artefacts of this nature, therefore, seem to be able to account for at least part of the variability observed with the red cell enzyme.

An additional explanation is provided by recent findings from Gopinath

and Vincenzi (*Biochem. biophys. Res. Commun.* 77, 1203; 1977) which confirm the existence in the red cell cytoplasm of large amounts of a polypeptide, possibly identical to the phosphodiesterase activator purified from brain tissues, which can bind reversibly to the internal surface of the membrane and interact with the red cell Ca pump in such a way as to produce a marked increase in the Ca affinity (and the V_{max} ?) of low-affinity enzyme preparations. The conditions which control the formation or breakdown of the activator-pump complex, both in the intact cell under physiological or experimental conditions and in enzyme preparations, are still poorly understood; and so is the potential physiological role of the activator as a regulator of Ca transport. But what seems clear is that the association is weak and reversible and can easily be altered by minor differences in the treatment of intact cells or membrane fragments thus inducing variability.

One can now apply this knowledge and also that derived from the characterisation of a phosphorylated intermediate of the red cell Ca pump (Schatzman & Burgin, *Ann. N.Y. Acad. Sci.*, in the press) to reinvestigate the properties of the (Mg+Ca)ATPase in microsomal preparations of different origins, this time by measuring the enzymic activity in the presence of ionophore and activator and by comparing the Ca phosphorylation product with that of red-cell membrane fragments. This should help establish whether failure of identification rather than genuine absence is the cause of the uncertainty surrounding Ca pump distribution. □



A hundred years ago

WE have received from Messrs. Eberstein, of Dresden, a specimen of an interesting "walking-stick for naturalists or tourists." The stick is a perfect *multum in parvo*, and contains quite a museum of scientific instruments. The handle alone contains a compass, a double magnifying glass, or pocket microscope, and a whistle. Below it there is a thermometer on one side of the stick and a sand-glass on the other. The body of the stick is partly hollow, and in its interior holds a small bottle, which is intended to contain chloroform or ether for killing insects. Along the outside of the body there is a half-metre measure, showing decimetres and centimetres. Near the end of the stick a knife-blade may be

opened, which serves for cutting off objects which cannot be reached by hand, such as aquatic plants, &c. At the extreme end a screw may hold in turn a spade (for botanists), a hammer (for geologists or mineralogists), a hatchet, or a strong spike, which would be of great use on glaciers. The whole is neatly finished in black polished wood.

DR. SCHLIEMANN is at Constantinople, and intends resuming his excavations in the Troad if he can obtain from the Porte fifty soldiers as a guard against robbers. From Berlin it is stated that a summary account of the German excavations at Olympia says that the number of marble objects found during the last three winters is 904; of bronzes, 3,734; of terra cottas, 904; of inscriptions, 429; and of coins, 1,270. All the more important ruins have been photographed, and the third volume of the official account is about to appear. An exhibition of all the casts taken will shortly be opened at Berlin.

From *Nature* 18, 1 August, 372; 1878.

review article

Functional specialisation in the visual cortex of the rhesus monkey

S. M. Zeki*

Anatomical and functional studies of the visual cortex of the rhesus monkey have shown that it is made up of a multiplicity of distinct areas. These seem to be functionally specialised to analyse different features of the visual environment.

THE cerebral cortex is the largest and most impressive part of the nervous system, in both monkey and man. There are several features that distinguish it from other parts of the nervous system. Perhaps chief of these is the enormous number of nerve cells that it contains¹. How this mass of nervous tissue is organised has been a subject of very considerable interest. For a long time, clinical neurologists believed that different functions, such as vision, audition, language, memory for words, memory for colours, and so on, were localised in structurally distinct regions of the cerebral cortex. The evidence for this was based partly on clinical studies from which it is rarely possible to know the exact location and size of the pathological changes in the cortex. It was also based in part on the anatomical method of cytoarchitectonics, which analyses how cells in different parts of the cerebral cortex are grouped into layers. Differences in the layering pattern of cells between different cortical regions were thought to reflect functional differences. This is undeniably so for some parts of the cortex. The structure of the motor cortex, to take one of the best known examples, is so different from that of the immediately adjoining sensory cortex, that it would be hard to imagine that this does not reflect the well known functional differences between the two areas. But such clear examples are the exception rather than the rule. Even determined students of cytoarchitectonics conceded that extensive regions of the cortex are of uniform cytoarchitecture^{2,3}. Of the remaining differences that they described, many were almost certainly the secondary consequence of the fissuration and foldings of the brain⁴.

Theories of functional localisation were, therefore, greatly compromised by technical difficulties and uncertain methods: hence they came to be questioned⁵. But they were not replaced by an alternative theory, based on more certain techniques. The problem itself thus remains, and is worth investigating with new methods. It can be made more manageable by restriction to a single system, such as the visual system, especially in an animal with a highly developed visual capacity, such as the rhesus monkey. One can then ask: how is the visual cortex organised to handle all the information available to it?

Considered cytoarchitecturally, the entire visual cortex of the rhesus monkey can be subdivided into two major zones (Fig. 1). Posteriorly, the striate cortex is so distinct and uniform that its borders are visible, in histological sections, even to the naked eye. The visual hemifields are completely mapped in the striate cortex^{6,7}. Most of its cells are responsive to contours of

specific orientation⁸ and although they tend to respond better to stimulation of one eye or the other, most of them can be influenced by stimulating either eye⁸. Two of the functions of the striate cortex, therefore, are the bringing of the inputs from the two eyes together and the detailed analysis of the visual fields for contour. Anterior to the striate cortex, and surrounding it, is another uniform field, of different cytoarchitectural design, known as the prestriate cortex (Fig. 1). Although the prestriate cortex has a very sharp boundary with the striate cortex, its anterior limits are very uncertain. Even its boundary with the inferior temporal cortex, a geographically separate area, but one well known from behavioural studies to be visual in function^{9,10} is uncertain. Because no clear cytoarchitectonic differences are evident over large extents of the prestriate cortex, much of it (for example Brodmann's area 18)³ was considered, mistakenly as we now know, to be a single cortical 'area'. Others proposed to include even further cortical regions in this single area⁵.

The only part of this entire visual cortex that receives a direct input from the eyes, through the lateral geniculate nucleus, is the striate cortex¹¹. Because of this arrangement of the visual projections, in monkey as well as in man, the striate cortex was referred to as the 'visuo-sensory' cortex². The prestriate cortex, not receiving a direct projection from the eye, was presumed to be the site of higher, associational processes and was called the 'visuo-psychic' band². Such a terminology implied that 'sensation' occurred at the level of the striate cortex and 'perception' at that of the prestriate cortex.

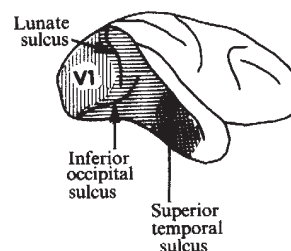


Fig. 1 Lateral view of the right hemisphere of the brain of rhesus monkey. The striate cortex (V1—vertical stripes) is situated posteriorly and ends 2 mm behind the lunate sulcus on the lateral side. The prestriate cortex (horizontal stripes) lies in front of the lunate sulcus and includes the depths of the deep sulci. Its anterior boundary is uncertain and it merges into the inferior temporal cortex (cross hatched), which is known also to have a visual function.

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Such theories were vague and ill formulated, especially when viewed in the light of more modern anatomical and functional evidence. This evidence has shown that the prestriate cortex, far from being the single cortical field that its cyto-architecture might suggest, is in fact composed of several distinct visual areas¹²⁻¹⁵. Each of these has its own specific anatomical connections and in each one the visual fields are separately mapped, in characteristic ways. Extensive mapping experiments have revealed that a multiplicity of visual areas exists in the cortex of other species of monkey as well¹⁶⁻²¹. It is obviously of interest to know the reasons for having so many different visual areas. But in order to investigate their functional characteristics, it is necessary to define their boundaries. This is a difficult task in a field of uniform cytoarchitecture. But it is a difficulty that must be overcome if one wishes to study functional localisation within the visual cortex. Fortunately, this can now be done.

Callosal connections as a guide to the visual areas

Because of the manner in which optic nerve fibres cross at the optic chiasm, it is the contralateral visual hemi-field that is represented in the striate area of each hemisphere^{6,7} and in each visual area of the prestriate cortex²²⁻²⁵ (Fig. 2). The visual field representation, separated into two hemifields by the chiasmatic crossings, is reunited in each visual area by the fibres of the corpus callosum^{15,26-29}. This is a massive commissure linking the two halves of the brain. Because the visual hemifields, including the vertical meridian, are separately represented in each area, the existence of several distinct patches of callosally connected prestriate cortex²⁹ is good evidence that there are several distinct visual areas. Each callosal patch belongs to the vertical meridian representation of a separate visual area. That this is indeed so can be shown directly by simple experiments which combine anatomical and physiological techniques^{15,27,28} (Fig. 3). The corpus callosum is sectioned six days before an electrophysiological recording experiment, a survival time which allows the cut callosal fibres to degenerate. Multiple electrode penetrations are then made in the prestriate cortex and the receptive field positions of cells in the different penetrations carefully noted. At the end of the experiment, the animal is killed and the brain sectioned and stained by a method which shows up the degenerated callosal fibres as well as the electrode tracks. It is found that cells lying in regions of the prestriate cortex where there is no degeneration and which, therefore, are devoid of callosal connections, have receptive fields distant from the vertical meridian. By contrast, cells in parts of the prestriate cortex which are callosally connected have receptive fields at, or extending to, the vertical meridian^{15,28}.

As the vertical meridian is represented at one or more boundaries of the visual areas in the prestriate cortex^{12,13,30,31}, the callosal patches also help in defining the boundaries of these areas. When recording from these areas, therefore, it is often sufficient to note the position of the electrode track with respect to the callosal degeneration to be able to state, with simplicity and certainty, which prestriate area one is recording from^{15,27,28,31}. But neither the boundaries of the areas, nor the callosal patches, bear any constant relationship to gross structural frontiers, such as the depth of sulci^{29,31}. Hence this approach of correlating positions of electrode tracks to the borders of the visual areas, seen through their callosal connections, is not a reprieve from the arduous task of making detailed reconstructions of the prestriate cortex for every recording experiment, but rather a confirmation that such reconstructions are mandatory.

This approach is also of great help in studying the connectivity of the different visual areas in the cortex. In such studies, one can inject labelled amino acids (which are taken up by nerve cells and transported to the terminals of their axons) into the prestriate cortex of animals whose corpus callosum had

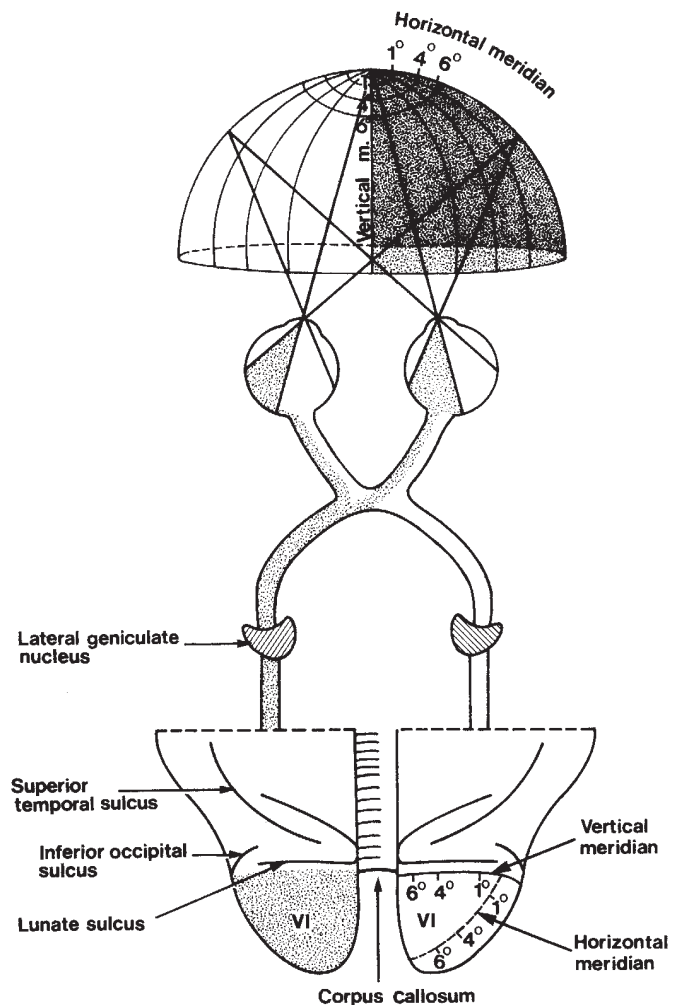


Fig. 2 Diagrammatic representation of the projections of the visual pathways, from retina to cortex, in the rhesus monkey. The temporal retina of the left eye projects to the left hemisphere, that of the right eye to the right hemisphere. The nasal hemi-retinas project to the contralateral hemispheres. Regions of vertical meridian representation in the visual areas of the cortex are callosally connected, but these are not shown in the diagram. Only the posterior part of each hemisphere, as viewed from above is shown.

been sectioned^{25,30,32}. One can then tell which cortical area the injection was made into and which cortical area the label appears in, merely by noting the site of injection and label distribution in relation to the bands of callosal degeneration defining the boundaries of the areas.

What else do these callosal patches tell us about the visual areas? Some of the callosal patches, such as the one at the V1-V2 boundary, are narrow while others, such as the one within V4, are broad^{29,31}. When such broad patches are reconstructed from section to section, one often notices a surprising variation in the density of degeneration within the callosal patch, with bands of heavy degeneration surrounding islands containing few or no degenerated fibres^{29,31}. As the callosal connections are indicative of vertical meridian representation, what is one to make of such a picture? Does it indicate further differentiation into more areas, or a set of sub-areas?

Definition of a visual area

There are at least five criteria which can be used to define a visual area. All these criteria can be applied to the striate cortex. They are (1) a well defined cytoarchitecture; (2) a complete map of the visual field; (3) a well defined anatomical input (in the case of the striate cortex an exclusive input from

the lateral geniculate nucleus); (4) distinct functional properties and (5) callosal connections.

Not all of these criteria can be used for identifying other visual areas, however. It is obvious that cytoarchitecture cannot be used to distinguish the prestriate areas from one another, as they all lie within a cortical region of uniform cytoarchitecture. Of the remaining criteria, the maps of the visual field are especially problematic. At the simplest level, one might consider that the visual field must be completely mapped once in a visual area. This is indeed so for the striate cortex^{6,7}. But in other areas, the same part of the visual field may be multiply represented, or only the central part of the visual field may be represented. These variations presumably depend upon the functions of the areas. Hence visual field maps do not form a constant criterion with which to define areas. Indeed, they can even be misleading.

Topographic maps in the cortex

A topographic map is a means of representing the body surface (in our case, the retina) in a certain order on the cerebral cortex. The order in which the retina is mapped on the cortical surface must bear a relation to the function of the area in question. Hence cortical maps are not all precise, geographic reproductions of the retinal surface, but are distorted in accordance with the functions of the areas. In the striate cortex, a proportionately larger amount of cortex is devoted to the fovea than to peripheral retina^{7,33}. This is not surprising. One of the major functions of the striate cortex is to analyse contours in the visual field⁸ and it is at the fovea that acuity is highest. Hence the need to distort the map in order to give the fovea greater and the periphery of the retina less representation, as in a distorting mirror. In spite of these distortions, continuity is maintained in the sense that adjacent points in the visual fields are represented at adjacent points in the striate cortex, which makes sense for an area analysing in detail orientations in visual space.

In V1, as well as in the two prestriate areas V2 and V3, the visual fields are separately mapped in a manner characteristic of the areas^{12,13}. The topography in all these three areas is predictable and, if one were to record from the posterior bank of the lunate sulcus, starting at the V1-V2 boundary and then proceed medially to the depth of the sulcus, and then laterally, receptive fields of successive cells would move from the vertical to the horizontal meridian and then back again to the vertical meridian (Fig. 3)^{12,13,28}. At the point of reversal, receptive fields suddenly change to larger (in V3) than in V2^{28,31}. In this instance, the reversal in the topographic map alone is indicative of two different areas although, of course, there is supporting anatomical^{12,25,30}, and electrophysiological²⁵ evidence to support such a delineation.

Reversals of this kind, when accompanied by changes in the properties of cells, or by differences in anatomical wiring, are acceptable evidence for boundaries between areas. But the functions of an area may dictate that the same part of the visual field must be multiply represented within it. In such an instance there would be several reversals in visual field representation within one area. There is therefore no reason to suppose that every reversal is indicative of a different visual area, nor that the visual fields need be mapped with the same degree of precision or distortion in areas with different functional properties. The motion area of the superior temporal sulcus, an area rich in directionally selective cells^{22,23} can be taken as an example in which the visual field map is organised in a different way and in which, unlike V1, V2 or V3, the same parts of the visual fields are multiply represented. Evidently its functions require this. In this area several reversals in receptive field positions sometimes occur even in a single penetration sampling tangentially from not more than 3 mm of cortex. Figure 4 shows this. Between cells 1 and 10, a total distance of less than 2 mm, receptive fields of cells move from a position 25° below and 25° contralateral to the vertical meridian (at 1) to the vertical meridian above the horizontal meridian (at 6), then

reverse to a position below the horizontal meridian (at 7), only to go up again at 11, and then move down once again at 20. Although the reversals are frequent, the functional properties of the cells do not change, all of them being directionally selective. It seems reasonable to consider it, therefore, as a single visual area in which the same parts of the visual fields are multiply re-represented and which has, therefore, a map of the visual field radically different from those of V1, V2, V3 and V3A—different because the functions of the area, in this case motion analysis, are different.

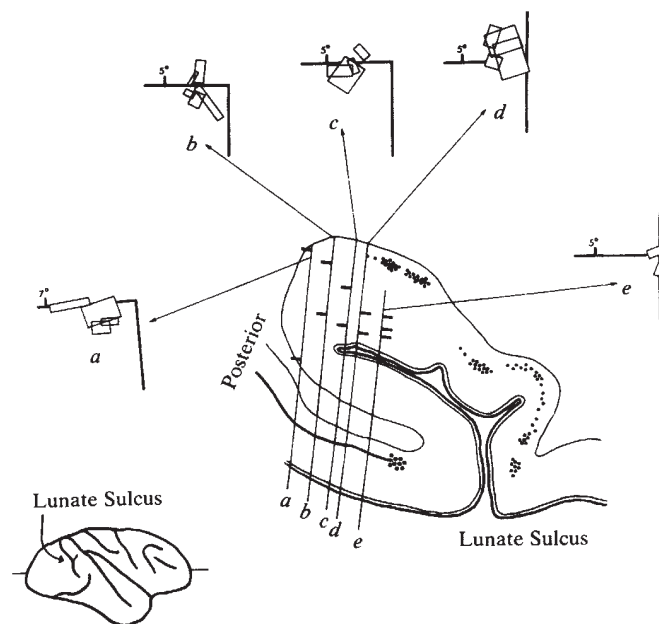
In both the motion area, and in V4 (ref. 31), the same part of the visual field, including the vertical meridian, is multiply represented. As regions of vertical meridian representation are callosally connected, it is hardly surprising to find that both have patchy and complex patterns of callosal connections, compared to those of V2 and V3 (refs. 29,31). The pattern of callosal connectivity is dictated by the manner in which the visual field is represented in an area. This in turn depends upon the function of the area. The function, finally, is dictated by the anatomical input. Hence, functional properties and anatomical inputs are the best criteria for defining an area.

Despite these qualifications, it is always best to consider all five criteria in deciding whether to confer the status of an area on a region of the visual cortex. An area such as V3A, for example, has a map of the visual field that is so predictable and different from other areas that one has to consider it as an independent area³¹, even if there is no compelling difference, to date, in the functional properties of cells between it and one of the two neighbouring areas, namely V3 (ref. 25).

The number of prestriate areas

Considering all the criteria given above, one can divide the prestriate cortex into at least six different areas (Fig. 5). All these areas, except the motion area of the superior temporal

Fig. 3 Reconstruction of five parallel horizontal penetrations through the lunate sulcus of a monkey whose corpus callosum had been sectioned 6 days before the recording experiment. The dots in the cortex represent callosal fibre degeneration. The penetrations were made at the level indicated on the drawing of the brain to the lower left. In the penetrations *a-e*, the short horizontal lines intersecting the electrode track indicate the region from which recordings were made. Receptive fields of groups of cells in each penetration are separately indicated in the plots *a-e*. Note that receptive fields of cells in regions where there is no degeneration lie away from the vertical meridian, whereas in regions of degeneration receptive fields are at the midline. (From ref. 28.)



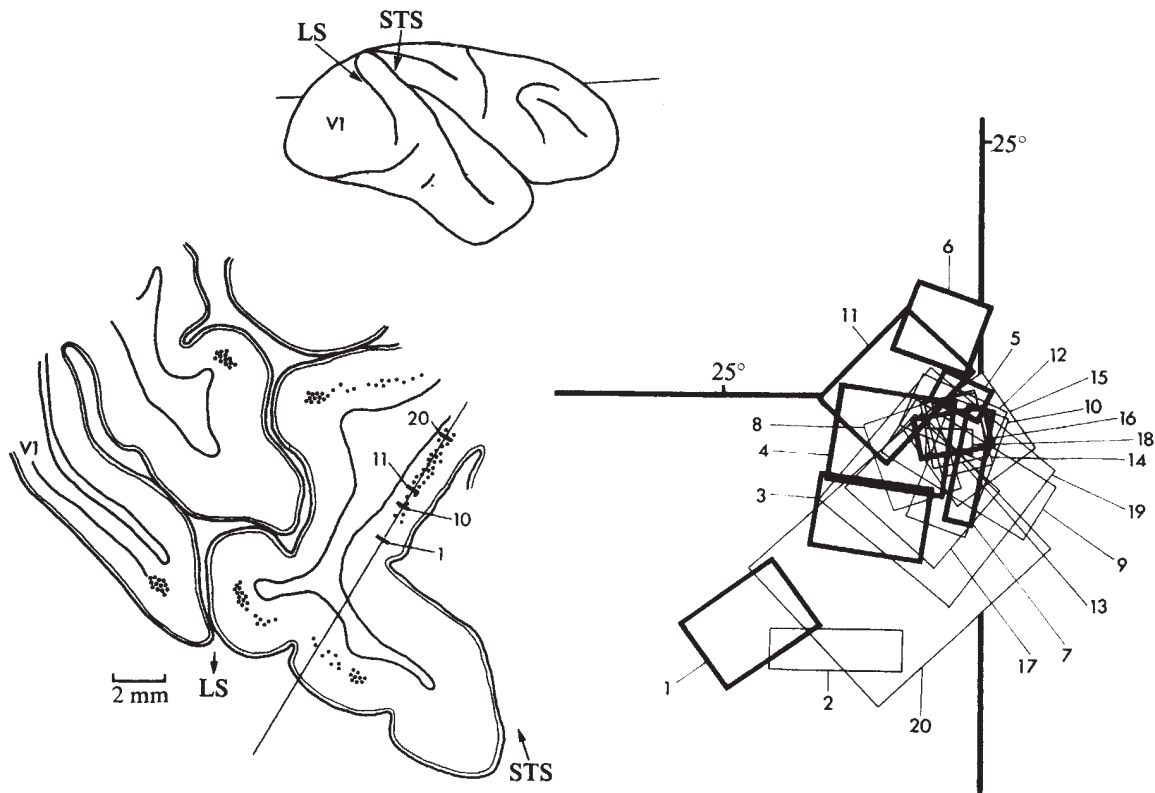


Fig. 4 Reconstruction of an experiment in which a long electrode penetration was made through the cortex of the medial part of the posterior bank of the superior temporal sulcus. To the left is a tracing of a horizontal section, taken at the level indicated on the surface drawing of the brain. The first 10 cells were situated in the region of the track enclosed by the two short horizontal bars, marked 1 and 10, intersecting the common electrode track and cells 11–20 were situated in the region marked by the corresponding horizontal bars. Conventions as in previous figures. The receptive fields of the individual cells are separately plotted to the right of the figure. Note that in the progression from 1 to 10, receptive fields move from the lower quadrant (1), to the centre of gaze and the vertical meridian (5), the superior quadrant (6) and then back down to the inferior quadrant (7), only to reverse again at 11, and yet again at 20. Note also that the first four cells, not being in a region of the cortex with callosal connections (as shown by the absence of degeneration) had receptive fields well away from the vertical meridian, whereas the remaining cells, being in regions of callosal degeneration, had receptive field extending to the vertical meridian. LS, lunate sulcus; STS, superior temporal sulcus.

sulcus, lie within Brodmann's cytoarchitectonic area 18 (ref. 3). Starting posteriorly at the border of V1, these are V2, V3 (refs 12, 13) and V3A (refs 25, 31), each one of which is identifiable as a separate visual area by (1) its distinct anatomical inputs; (2) its separate callosal connections; and (3) the separate, distinctive and topographic map of the visual field within each area. As well, although most cells in V2 and V3 are orientation selective³⁴, V3 cells have larger receptive fields than V2 cells at comparable eccentricities^{28,31}. This difference alone enables one to state with certainty the transition from V2 to V3 in a single penetration. It is, however, more difficult to distinguish between V3 and V3A on the basis of the properties of single cells, as studied to date²⁵. Most cells in both areas are orientation selective and there is no sharp difference in receptive field sizes at equivalent eccentricities, as there is between V2 and V3²⁵. Nevertheless, the different, and distinctive map of the visual field within V3A, compared to other areas, as well as its distinctive anatomical input²⁵ and callosal connections^{25,31}, make of it an independent visual cortical area.

Lying lateral to V3A in the lunate sulcus, and extending on to the prelunate gyrus, and then the lateral part of the posterior bank of the superior temporal sulcus is a zone of cortex, which can be subdivided into a posterior area with its distinctive anatomical and callosal connections¹⁴ and an anterior area, which also has its separate anatomical and callosal connections⁵. The visual fields are separately mapped in each area in a complicated way, the same part of the visual field being multiply represented within each area. Because the properties of the cells in the two areas are so similar (see below) I group them

together as the areas of the fourth visual complex^{15,34} (V4). The reason for not naming these areas further is that details concerning their common borders remain to be settled. There are suggestions from the anatomy that it may be possible to subdivide each area^{14,15}.

Lying more medially within the posterior bank of the superior temporal sulcus is another visual area, recognisable as an independent area by its distinct anatomical and callosal connections and by its distinct functional properties^{22,23}. For the present, I refer to it as the motion area.

Functional specialisation within the visual cortex

The callosal landmarks allow one to overcome the problem of defining the boundaries of the visual areas. One can now record from single cells in these areas and compare the response of cells in one area with those in another. Fortunately, there are no obvious differences in the properties of single cells when one compares recordings made from intact brains with recordings made from brains with a sectioned corpus callosum^{15,25}. Hence sectioning the corpus callosum provides useful anatomical guides to the borders of the cortical areas without interfering with the properties of single cells.

Recordings made from 1,500 single cells in the prestriate cortex³⁴, summarised in Fig. 6, reveal differences in the distribution of orientation, colour, and motion selective cells in the different cortical areas. Most orientation selective cells are grouped into three areas—V2, V3 and V3A. Significantly,

receptive field sizes of cells are smaller in V2 than in V3 or V3A at equivalent eccentricities^{28,31}. Colour specific cells (that is, those responding to one part of the spectrum and not to other parts or to white light, or those excited by one part of the spectrum and inhibited by another) are grouped into the areas of the V4 complex³⁴. The percentage of orientation selective cells in the areas of the V4 complex is significantly lower than those in V2, V3 or V3A. Finally, the directionally selective cells (that is those responding to motion of the visual stimulus in one direction only) are found in heavy concentrations in the motion area of the superior temporal sulcus³⁴. Even orientation selective cells here are also directionally selective²². This positive evidence is as compelling as the negative evidence. For example, there is no hint of colour coded cells in V3 or V3A, or in the motion area, and only a small proportion of directionally selective cells in V2, V3, V3A or the areas of the V4 complex. It is obvious that these different areas do not analyse the same features of the visual environment at an ever increasing complexity, but rather that they analyse different types of information in the visual environment.

Paradoxically, these very differences in the type of information analysed by the different prestriate areas must impose certain similarities on them. If different prestriate areas are to analyse different types of information contained in the same part of the visual field, the same part of the visual field must be represented in each area. This is indeed the case. But one would not expect the same extent of the visual field to be mapped in each area. There would be little reason to map the far periphery of the visual field in an area which is concerned with analysing colour in the visual field since colour perception is best in the central 20° and absent in the far periphery. By contrast, there is every reason to represent the peripheral visual field in the motion area since displacements in the peripheral field are powerful visual stimuli. Hence the failure to identify any representation of the visual field beyond 20° in

V4^{15,24}; hence also the powerful representation of the periphery in the motion area of the superior temporal sulcus (S.M.Z., unpublished). Variations in the extent of the visual field represented in different prestriate areas have also been reported for other species of monkeys²¹. Hence the differences in function between the different areas are superimposed upon common features. Anatomically, the common feature is a uniform cytoarchitecture upon which differences are superimposed by different anatomical inputs³². Functionally, the common feature is the re-representation of the same part of the visual fields and of the two eyes in every area³⁴, the differences being that in every area a different type of information is analysed. Thus our present-day knowledge of the organisation

Fig. 6 The percentage distribution of orientation selective (a), colour selective (b) and directionally selective (c) cells in the prestriate areas.

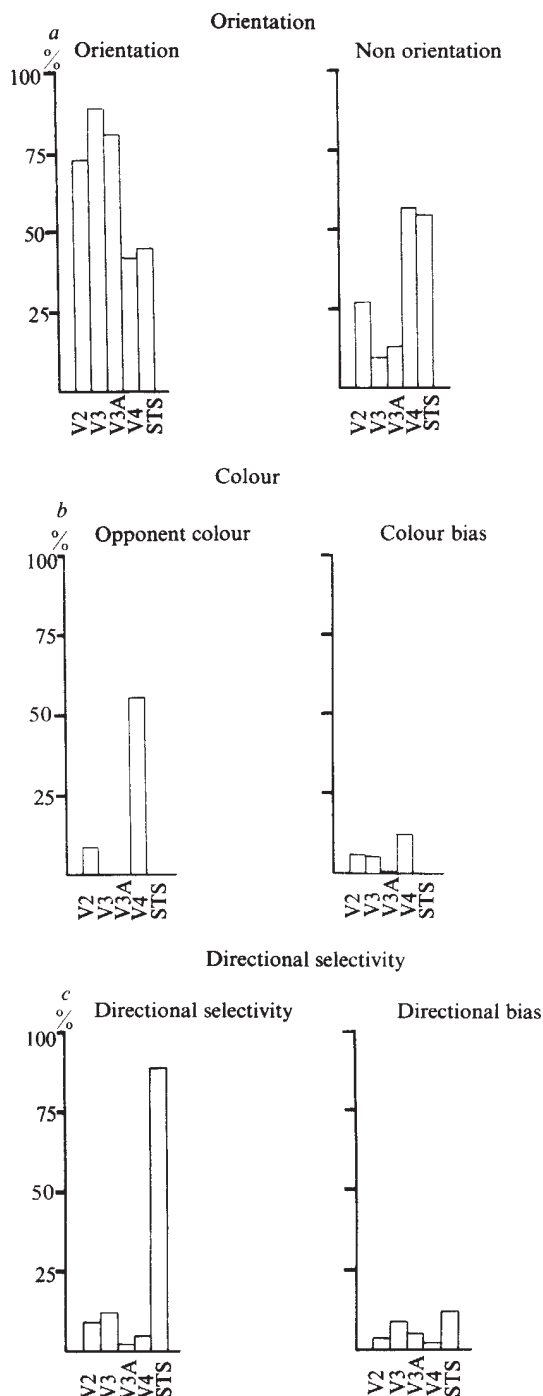
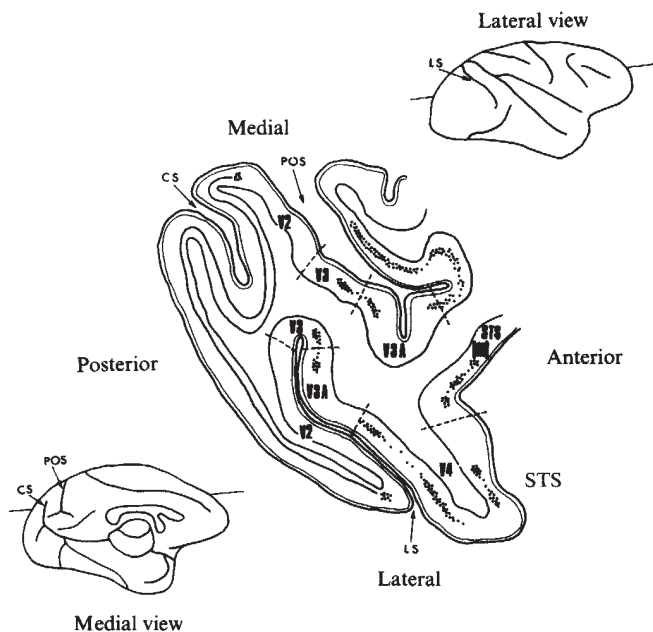


Fig. 5 A tracing of a horizontal section through the striate and prestriate cortex, at the levels indicated on the medial (left) and lateral (above, right) surface drawings of the brain of an animal in which the corpus callosum had been sectioned. The degeneration following such a section is represented as dots. Note that the callosal patches can be narrow (as at the V1-V2 boundary) or broad and patchy (as in V4) and in the medial part (STS M) of the superior temporal sulcus. The callosal landmarks indicate that several distinct visual areas must exist within a single cyto-architectonic field. The boundaries of the areas are indicated by interrupted lines. Continuous line in the cortex represents V1. LS, lunate sulcus; STS, superior temporal sulcus.



of the visual cortex goes some way to support theories of cortical localisation of function which the early neurologists fought so hard to establish³⁵. It would, of course, be naive to suppose that the kind of functional specialisation revealed in these studies implies that every visual area has one function only. Future, more sophisticated, studies may well reveal other functions for each visual area.

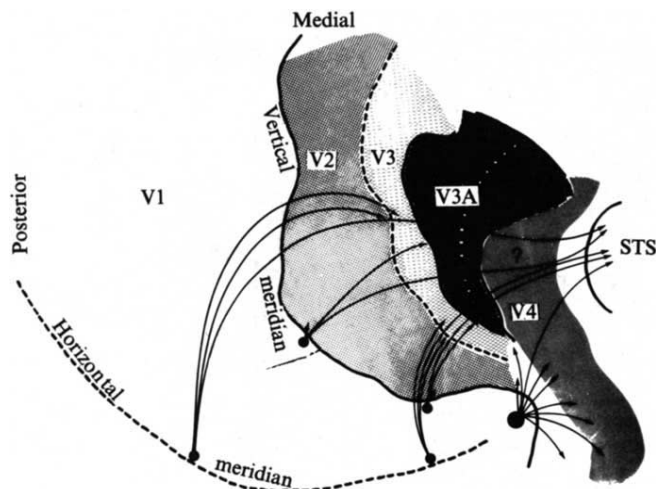
What does this kind of anatomical and functional organisation reveal about the cortical processes involved in vision? It tells us that the theory of a visuo-sensory cortex limited to the striate cortex, and a visuo-psychic band incorporating the prestriate cortex is almost certainly false, because the sensory analysis of the visual environment is far from complete at the level of the striate cortex. It tells us, instead, that there are several strategies that the cortex utilises concurrently in analysing the visual environment. One strategy is for each area to act not only as an integrator of the information coming to it, but also as a distributor of information, sending different types of information to different visual areas for further analysis^{27,32}. Nowhere is this more evident than in the striate cortex. All the information in the retino-geniculo-cortical system passes through the striate cortex. The striate cortex, in turn, sends distinct outputs to different prestriate areas^{27,32} (Fig. 7). It would be difficult to imagine that it sends the same information to these different areas. Differences in the properties of cells in the different prestriate areas to which it projects confirm that it does not. But it must not be imagined that this information is necessarily relayed in a passive way. Rather, much of the information, but perhaps not all, is elaborated before being relayed. For example, the striate cortex combines the information coming from the two eyes⁸. It also uses the information provided by the centre-surround cells of the lateral geniculate nucleus to provide information about orientation⁸. It then passes this information on orientation selectivity to other prestriate areas, whose cells are mostly binocularly driven³⁴, and generalises it over greater or lesser extents of the visual field in the different prestriate areas. Such a double role of integration and distribution may also be postulated for other prestriate areas, as each prestriate area, in turn, projects to more than one further area¹⁴.

This strategy of analysis and integration of information, and the distribution of information, tells us of two further strategies that the visual cortex uses in analysing the visual environment. One is the simultaneous analysis of different types of visual information in the different areas—hence the need to represent the visual field separately in each area. Another strategy is a hierarchical one, the information analysed in one area being used in another, more central, area in a more complex fashion. For example, the truly binocular, orientation selective cells of V2³⁴ use the information provided by the orientation selective cells of V1 which, however, are not truly binocular, but prefer one eye⁸. In this hierarchical system, receptive fields of cells in V2 are larger than those in V1, at comparable eccentricities, just as the fields of cells in V3 (which also receives a direct input from V1)^{12,13} are larger than those in V2. In such a progression, orientation selectivity is generalised over ever wider parts of the visual fields.

The picture of the visual cortex that we have today, then, is one of a large cortical field containing several different visual areas. These have some features in common such as analysing the same parts of the visual fields, and yet differ in being functionally specialised to analyse different features in the same part of the visual field. Our picture of the prestriate cortex is obviously far from complete. Much of it, especially on the medial side of the brain, remains unexplored and there are powerful hints from anatomical studies that yet further distinct visual areas may be found^{14,15,36}. Moreover, the details of how an area, such as V4, analyses information relating to colour remain far from settled. Questions also remain concerning functional specialisation for other attributes, such as depth detection^{37,38}. Finally, one is naturally very curious to learn how the information analysed in different areas of the prestriate cortex is then used to give a unitary 'perception' of the visual world. Much exciting work remains to be done, therefore, and to this extent the work done to date is no more than a beginning. But it is gratifying to know that by using relatively simple anatomical and electrophysiological techniques one has every hope of beginning to acquire an understanding of what is undoubtedly the most mysterious and interesting part of the body—the cerebral cortex.

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Fig. 7 Summary diagram of the projections of the striate cortex, displayed on a 'flattened out' striate and prestriate cortex. Only the projections of the part of the striate cortex representing lower visual fields are shown, but the part of the striate cortex representing upper visual fields has similar projections. Interrupted lines indicate the representation of the horizontal meridian, continuous lines that of the vertical meridian. Note that each part of the striate cortex has direct projections to V2, V3 and the medial part of the posterior bank of the superior temporal sulcus (STS). The region of the striate cortex at which the centre of gaze is represented has an additional projection—to V4. (From ref. 34.)



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articles

Regional structure and geodynamics of the upper mantle beneath the Massif Central

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A systematic mapping of the deformation structures in peridotite xenoliths from volcanic occurrences suggests an upper mantle diapiric rise beneath an area of crustal thinning in the Massif Central, France. The uprise would have been initiated 5 Myr ago.

MOST studies of peridotite xenoliths in basalts have previously been carried out with the idea of obtaining very general reconnaissance data about the nature of the upper mantle in terms of its composition, mineralogy, structure and geophysical properties. The only study detailed enough to give information at a regional level is that of Jackson¹ on the nature of the upper mantle beneath Hawaii. As far as the French Massif Central is concerned, many petrological², geochemical³ and structural⁴ studies have been undertaken to understand better the nature of the upper mantle situated beneath this volcanic province. Considering the abundance of xenolith occurrences and their favourable distribution throughout the Massif Central, we thought that a very comprehensive study of these rocks could increase our knowledge of the upper mantle by giving direct evidence about its heterogeneities on the regional scale. A recent geophysical synthesis⁵ suggested that an ascent of the upper mantle and lower asthenosphere is situated directly beneath the centre of the Massif Central (Fig. 1). Here we investigate whether or not this geophysical anomaly corresponds to a geodynamic anomaly in the mantle, expressed by an observed geographical division of textures and other parameters of deformation, such as stress and strain rate, in peridotite xenoliths. Our data are based on 1,650 xenoliths, collected from 70 volcanic occurrences; 850 thin sections were studied and 600 microprobe mineral analyses carried out. Finally, 100 measurements of textural parameters have enabled us to estimate stresses and strain rates. Complete analytical data are presented in table form elsewhere⁶.

Geographical distribution of textural types

The classification of xenolith textural types used here is derived from that of Mercier and Nicolas⁷, with two exceptions. Here, we use the term 'coarse grain' in place of 'protogranular', and 'mosaic' instead of 'equigranular', following the nomenclature of Harte⁸. We have noted three principal textural types: (1) coarse-grained texture (4 mm) indicating little or no deformation. (2) Porphyroclastic texture, indicating a higher degree

of deformation. Two generations of olivine crystals coexist: larger porphyroclasts and smaller, recrystallised neoblasts: an olivine foliation and spinel lineation develop. (3) Mosaic texture, indicating a high degree of recrystallisation; grain size is 1 mm. Porphyroclasts tend to disappear as neoblasts are formed at their expense. When recrystallisation is complete, we arrive at the granuloblastic type of Harte⁸, which is rare in the Massif Central.

In addition to these three main textural types, poikilitic and coarse-grained tabular textures are less frequently observed⁶⁻⁹. These two textures, which are associated with the deep, central portions of xenolith occurrences have several identical characteristics, suggesting a common origin perhaps due to magmatic accumulation (E. Berger, personal communication).

At each collection point, the population of xenoliths has been studied according to the textural classification mentioned above. From this, we can extract systematic percentages forming the basis of a statistical study (Table 1).

A single volcanic vent usually produces xenoliths of one dominant textural type, accompanied by a smaller number of xenoliths in continuous textural relation with it. Occasionally, we observe the coexistence of a continuous series, from coarse-grained to mosaic textures, in the same outcrop. This intermixture of extreme types is, however, the exception to the rule.

Geographical distribution of textures is not random. It is ordered from the centre towards the periphery of the Massif Central such that the most internal occurrences exhibit dominantly mosaic textures whereas the external ones are predominantly coarse grained (Fig. 4). The peripheral district of Coirons, south-east of the Massif Central, is an exception to this centred distribution; it has xenoliths of both porphyroclastic and mosaic textures.

Table 1 Statistical study of the textural types in the Massif Central

Texture	Fraction of studied population in Massif Central	Fraction of volcanic occurrences where present	Mean fraction in these occurrences
Poikilitic and tabular	2%	15%	13%
Coarse grained	41%	54%	67%
Porphyroclastic	16%	50%	31%
Mosaic	41%	69%	60%

Texture distribution as a function of volcanism age

Peridotite xenolith occurrences in the Massif Central result essentially from plateau basalt volcanism along with that of the Pliovillafranchian episode, 1–4 Myr ago. However, some volcanic episodes are older, dating from 7 to 17 Myr ago. These include volcanics in the Sioule, the Limagne and the Causses districts which have all produced coarse-textured xenoliths, either in exclusive or dominating assemblages. At the periphery of the Massif Central, coarse-grained xenoliths are equally associated with recent volcanics, such as those of the Vivarais, or with somewhat older ones. Mosaic-textured xenoliths are only present in relatively recent volcanic provinces: 5.5 Myr in the Coirons¹⁰ 4 Myr in the Cantal and less in the other districts.

Estimation of stresses and strain rates

Deformation studies in metals¹¹ have shown that two structural piezometers are applicable to natural deformations: the size of olivine substructures, and the grain size of neoblasts. The latter parameter provides the most reliable measure of stress as, unlike olivine substructure, it is much less sensitive to late-stage stresses and static recovery¹².

Many calibrations for grain size and substructure dimensions have been proposed^{9,13–15} but here, we use the relationships of Mercier⁹:

Size of substructure: $\sigma = 115/d$ (σ = kbar; d = μm)

Grain size:

$$\sigma = 1.091d^{(-0.714)} \exp(4853/T) \quad (\sigma = \text{kbar}; d = \mu\text{m}; T = \text{K})$$

For the reasons given above, we consider stress estimations calculated on the basis of neoblast size to be more accurate. However, stress is calculated for coarse-grained textures on the basis of substructure size, because the grain in this case is the original grain and not that of the neoblasts, therefore, the calculated value would not be indicative of the real rock stress. Calculated stress values range from 300 to 700 bar for mosaic textures and from 300 to 400 bar for coarse-grained textures. These values are comparable with an upward thrust of 500 bar created by a mass deficit at depth, as suggested by the model of Perrier and Ruegg⁵ (Fig. 1).

A certain number of porphyroclastic textured xenoliths indicates stresses of 400–700 bar, about the same value as for the other xenoliths. Some porphyroclastic textures have a stress value that extends from 700 bar to >1 kbar. They are also

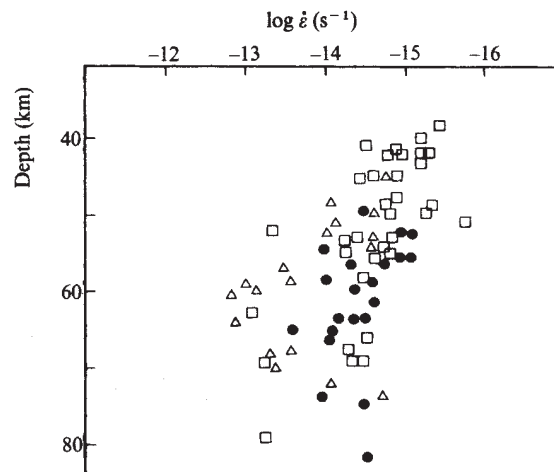


Fig. 2 Strain rates ($\dot{\epsilon}$) as a function of depth in the mantle for the various textural types: ●, coarse grained; △, porphyroclastic; □, mosaic.

located in a 1-km thick band along the margin of the deformed area in Fig. 4. These two different porphyroclastic textures may result from different flow conditions. The first one, representing a transition between coarse-grained and mosaic textures, following the textural cycle of deformation of Mercier and Nicolas⁷ might indicate steady state flow in the mantle. The second type of high stress porphyroclastic textures might be closely associated with movement in mantle shear zones.

Strain rate

Strain rate is related to stress by the following creep law¹⁶.

$$\dot{\epsilon} = \text{Cte } \sigma^n \exp -(Q + PV)/RT$$

where $\dot{\epsilon}$, is strain rate; σ , stress; Q , activation energy; P , pressure; T , temperature; V , activation volume; R , perfect gas constant.

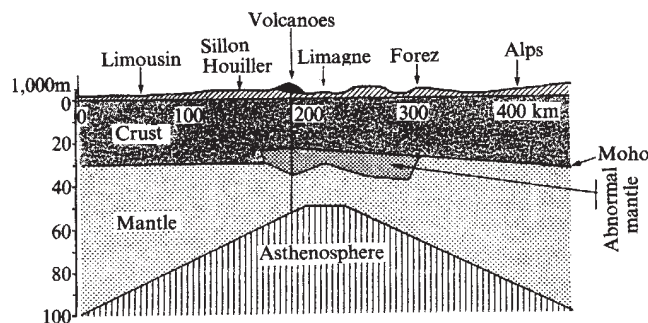
Here, we use the coefficients of Mercier⁹: $\text{Cte} = 1.7 \times 10^9$; $n = 3.2$; $V = 262.8 \text{ cal kbar}^{-1} \text{ mol}^{-1}$ and $Q = 13,500 \text{ cal}$.

Coarse-grained and mosaic textures give calculated strain rates of the order of 10^{-15} – 10^{-14} s^{-1} . Calculated strain rates for porphyroclastic textures range from 10^{-14} to 10^{-13} s^{-1} (Fig. 2). Those nodules deformed at an elevated stress value also have strain rates 10 times higher. These xenoliths may correspond to areas in which principal movements are concentrated.

Pressure–temperature equilibrium conditions

The geobarometer–geothermometer used here is that of Mercier¹⁷. The majority of xenoliths seem to have been equilibrated between 12 and 25 kbar (at depths ranging from 40 to 80 km) and between 920 and 1,100 °C (Fig. 3). A few rare samples record conditions up to 30 kbar (95 km) and 1,220 °C. This may be due to the particular mineralogy of the rock (for example, the garnet therzolite of Eglazines^{4–18}) or due to a cumulate origin (for example, coarse-grained tabular or poikilitic textures).

Fig. 1 Geophysical section of the crust and upper mantle beneath the Massif Central after Perrier and Ruegg⁵.



These pressure-temperature data determine geotherms with slopes close to $13.5^{\circ}\text{C kbar}^{-1}$ (or $4.2^{\circ}\text{km}^{-1}$), between 12 and 20 kbar. This type of geotherm is characteristic of 'tectonically active regions', that is, ridges or hot spots. Xenolith textures are not clearly arranged on the *PT* diagram. However, mosaic textures do indicate pressures generally less than 17 kbar whereas coarse-grained textures correspond to pressures greater than 16 kbar.

Discussion

This discussion has aimed to propose a geodynamic model of the upper mantle beneath the Massif Central, based on the study of peridotite xenoliths brought up by basalts. With this in mind, we must specify the original spatial coordinates of the xenoliths in the mantle, as well as their temperature at the moment of incorporation into the basaltic magma. Horizontal location of the xenoliths in the mantle is not a real problem, as most of the nodules come from cinder cones, which have a vertical projectile path. On the other hand, determination of temperature and pressure (depth) conditions is more complicated. It necessitates the use of pyroxene geothermometers and geobarometers as well as the supposition that an actual equilibrium state exists between the different phases present.

Therefore, we must first consider the controversial question concerning the validity of pyroxene geothermometers and barometers. Then we must discuss the problem of xenolith equilibria modifications resulting from contact and interaction with a basaltic magma during passage through the Earth's crust. This contact could have also provoked static recovery, modifying the dislocation structures used in the determination of stress values. Finally, we must remember that we are integrating data on xenoliths erupted between the Miocene and the present. During this time lapse, the mantle could have been susceptible to many important modifications.

After an initial euphoric period we must consider it premature to assign equilibrium pressures to spinel peridotites based on coefficients of aluminium partition in pyroxenes. On the other hand, different geothermometers exist which measure apparently correct temperatures.

An estimation of the xenolith depth of origin is essential, we have used the geothermometer-geobarometer couple of Mercier¹⁷. Its experimental and thermodynamic evidence can be criticised as much as other models, but it has as an advan-

tage the possibility of empirical corrections from which reasonable pressure estimates can be made. Pressure controls can therefore be obtained based on the presence of the aluminous phase in peridotite (garnet on the high pressure side and feldspar on the low pressure one). Finally, these estimates of pressure or depth must be considered as very approximate; relative values are probably correct.

Cross cuts through xenoliths show that chemical contamination by basalt is uncommon or, at most, limited to the exterior of the nodule. Even so, higher temperature and lower pressure in the basalt compared with the original xenolith environment could modify element equilibria between coexisting phases. Coisy⁶ shows that although this effect exists it can be accounted for if enough mineral analyses are available in the considered xenolith.

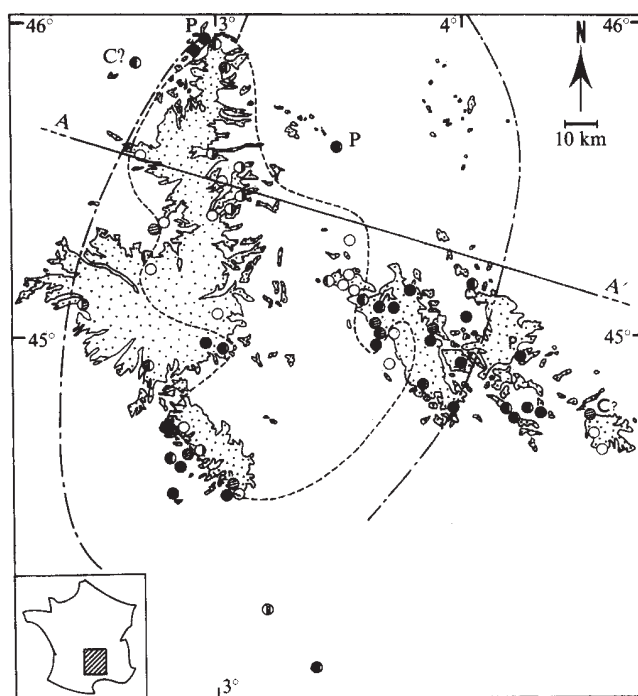
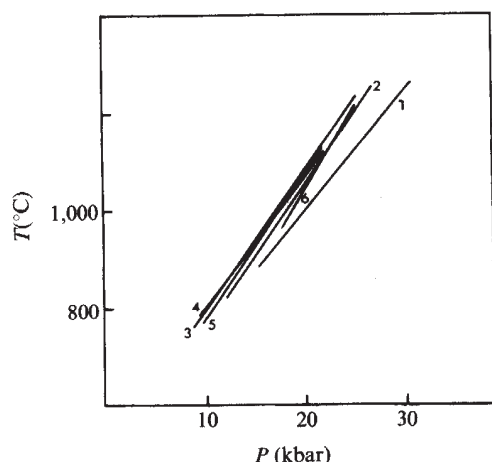


Fig. 4 Distribution in map of the various textural types of mantle xenoliths. ●, Coarse grained; ◐, porphyroclastic; ○, mosaic; P, poikilitic; C, cumulate; (---), contours of presumed diapir; (—), contours of abnormal mantle and thin crust.

Fig. 3 Geotherms for the different districts in Massif Central. 1, Livradois; 2, Céallier; 3, Cantal; 4, Aubrac; 5, Devès; 6, Velay.



Deformation structures in xenoliths could have been modified by static recovery due to a temperature of $1,200^{\circ}\text{C}$ or more in the basaltic magma. Also shock incurred from a violent explosion during xenolith rise and eruption could have introduced a new population of dislocations. In a systematic study of dislocation structures in basalt xenoliths, Gueguen¹⁹ showed that certain peridotites present deformation bands operating with low temperature slip systems, superimposed locally on a dislocation structure of high temperature and low stress, due to mantle creep. He attributes the origin of these bands to mechanical effects during eruption. These effects can be compared with the other dislocations and correspond to very weak deformation ($<1\%$). They certainly do not impede the study of dislocation structures developed during major creep in the mantle. The effect of static recovery on olivine dislocation structures is being studied by Ricoult. The preliminary results indicate that it has no influence on the olivine neoblast size and little influence on the size of subgrains disorientated by more than a degree which are considered here.

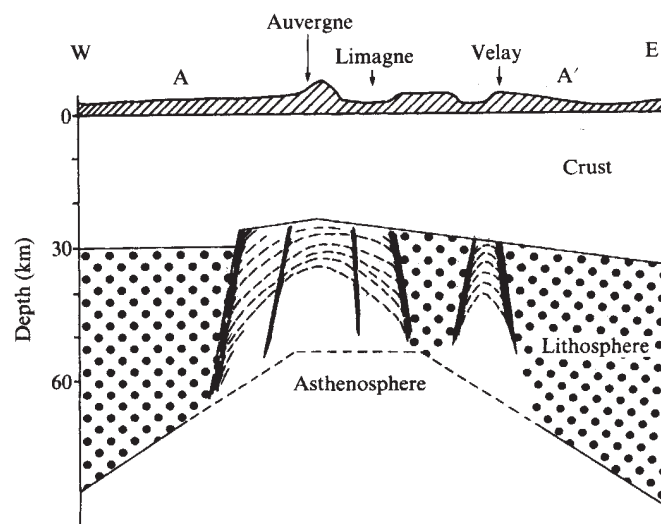
Xenoliths show the state of the mantle existing at the moment of their extraction. This would correspond to ages, in the Massif Central, between the Miocene (20 Myr) and the present. We can narrow this time lapse to the past 4 Myr as the bulk of volcanic activity is restricted to this latter period.

It is important to know how the mantle could have modified itself structurally, chemically and thermally during this time. In other words, is it valid to integrate into a single model of the mantle information on different mantle states between 4 Myr ago and the present, including the geophysical data of Figs 1 and 4 which illustrate a present day situation?

Large horizontal movements in the mantle beneath the Massif Central related to the genesis of a rift zone ought to be excluded considering the modest amount of actual surficial rifting noted. Thus, the transport of material and heat by convection should be restricted to the vertical movements suggested by Perrier and Ruegg's model⁵, corresponding to vertical distances of about 12 km. Calculations on heat transport by conduction alone show that in 4 Myr a thermal wave only progresses about 3 km in the mantle. These estimates agree with our data on the distribution of temperatures in time (Fig. 3). The geotherms constructed for different districts in the Massif Central are identical and of uniformly high temperature, with the exception of the Livradois (Montboissier) geotherm. It is both colder and older (13–17 Myr)²⁰ than most other districts. Here, we can suppose that a mantle heating episode occurred between these two disparate ages also expressed by increasing volcanic activity. Since 4 Myr ago no reversals have appeared in this heating trend. In fact, the Vivarais district of near present age continues to exhibit a high temperature geotherm¹⁸.

Textures showing considerable plastic deformation of the mantle only appear in more recent xenolith occurrences. Coarse-grained xenoliths, which indicate little or no deformation, however, do not necessarily exhibit a preferential age; we conclude that: (1) the normal structure of the lithospheric mantle (not deformed) is probably coarse grained; (2) creep in the mantle beneath the Massif Central was instigated perhaps, 5.5 Myr ago and certainly by 4 Myr ago. This probably corresponds to the heating episode just considered. The presence of coarse-grained textures in very recent xenolith occurrences may be due to random sampling of mantle regions having escaped previous deformations.

Fig. 5 Profile of diapiric intrusion taking into account the geographical distribution of peridotite xenoliths textural types (AA' in Fig. 4 provides approximate location). This profile integrates the geophysical model of Fig. 1. ●, Coarse grained; = = =, mosaic; —, porphyroclastic.



Geodynamic models

Textures resulting from large plastic deformation in the mantle during the past 5 Myr are limited geographically to the centre of a core zone within the Massif Central. This central core is characterised by crustal thinning and an abnormal mantle, as defined by geophysical parameters (Fig. 4). Around the limit of the very deformed core area, textures are distributed in a regular way, expressing a decreasing gradient of deformation from porphyroclastic textures in the centre to the coarse-grained textures furthest away from the core (Fig. 4).

Analysis of stresses has shown two textural populations. The first includes xenoliths characterised by any of the three principal textures, corresponding to stresses of 250–650 bar. The other is composed of porphyroclastic textures, corresponding to stresses >700 bar. This latter group of xenoliths is distributed along a band about 1 km wide at the limit of the deformed core zone shown in Fig. 4. Analysis of mantle strain rates show that rates of 10^{-15} – 10^{-14} s⁻¹ are typical for moderately stressed nodules. For porphyroclastic textures deformed at higher stresses, the strain rate rises to 10^{-13} s⁻¹.

From this, we conclude that textures of the first group correspond to regional mantle flow. The porphyroclastic textures developed at elevated stresses, on the other hand, indicate the existence of a mechanical instability, concentrating deformation around the periphery of the deformed core zone. If these bands of instability were orientated vertically, they would contribute to an ascent of the mantle at a rate of 1–5 mm yr⁻¹, giving 1–5 km of vertical mantle rise in 5 Myr. Such structures would certainly help to explain an important part of the total picture of mantle rise, envisaged by Perrier and Ruegg (Fig. 1).

These different data allow us to propose a geodynamic model of recent (<5 Myr) mantle rise, poised beneath the core of the Massif Central (Fig. 5). Based on the distribution of xenolith textures in time and space, the model agrees with the geophysical results both for the location of the diapir and for the stress and strain rate estimates. This mantle rise may be the result of a poly diapir, because of the lobate contours of the limits of intense deformation. Instead of one single large diapir (50 by 100 km) we imagine a structure resulting from the conjugate action of small, adjoining diapirs (20–30 km diameter maximum). The peripheral district of deformed xenoliths of Coirons (south-east of the Massif Central) would be poised above a small independent diapir, situated on the eastern border of mantle rise.

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Crystallographic studies on the activity of glycogen phosphorylase *b*

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*High resolution studies on the crystal structure of glycogen phosphorylase *b* have identified the catalytic site to which the substrate glucose-1-phosphate binds strongly with some local conformational changes. The site is situated 8 Å (phosphate-to-phosphate distance) from pyridoxal phosphate, an essential cofactor of all glycogen phosphorylases. The catalytic site is 33 Å from the site in the N-terminal portion of the molecule to which adenine nucleotides bind. In contrast to phosphorylase *a* (the active form of the enzyme which is phosphorylated at Ser 14), the positions of the first 19 residues of phosphorylase *b* are not well defined.*

SINCE the fundamental work of Cori and Cori¹ glycogen phosphorylase (EC 2.4.1.1) has represented an outstanding example of an enzyme whose activity may be modulated either by covalent modification or by noncovalent binding of metabolites. The enzyme exists in two interconvertible forms. Phosphorylase *b*, found in resting muscle, requires AMP (or IMP or certain analogues of AMP) for activity and is inhibited in an allosteric manner by ATP, ADP and glucose-6-phosphate. The levels of these metabolites in resting muscle are such that the enzyme is inactive. In response to hormonal or nervous stimulation, phosphorylase *b* is converted to phosphorylase *a* by the action of phosphorylase kinase through phosphorylation of a single amino acid (serine 14) per monomer. Phosphorylase *a* is active in the absence of metabolites. It no longer requires AMP for activity (although AMP can produce an enhancement of activity) and does not exhibit allosteric inhibition by ATP, ADP or glucose-6-phosphate, although it is strongly inhibited by glucose. The properties of the enzyme and its control have been reviewed².

Independent crystallographic studies are underway on phosphorylase *b* in Oxford³⁻⁵ and phosphorylase *a* in Edmonton⁶⁻⁸ with the aim of providing a structural explanation for the catalytic and control properties of the enzyme. The crystals of phosphorylase *b* are grown in the presence of 2 mM IMP, a weak nucleotide activator⁹, and those of phosphorylase *a* are grown in the presence of 50 mM glucose and contain an inhibited form of the enzyme. Both structures have now been solved at 3 Å resolution^{5,7}, and these studies and metabolite binding experiments^{7,8,10} have provided information concerning the fold of this large polypeptide chain and the location of binding sites. The complete sequence for the 841 amino acid

residues of phosphorylase has recently been determined in Seattle¹¹ and this outstanding achievement provides a most significant advance in our understanding of phosphorylase.

We describe here the structure of phosphorylase *b* and its complexes with glucose-1-phosphate, a substrate molecule, and with maltotriose, an analogue of the glycogen substrate.

X-ray analysis

The crystallisation of phosphorylase *b* in the presence of 2 mM IMP has been described⁴. The crystals are tetragonal, space group $P4_32_12$, with unit cell dimensions $a = b = 128.5$ Å, $c = 115.9$ Å. The asymmetrical unit contains one phosphorylase subunit (molecular weight 97,000). The two subunits of the functionally active dimer are related by the crystallographic twofold axis at $z = \frac{1}{2}$. The heavy atom derivatives used for the 3-Å studies were the same as those used at low resolution, namely ethylmercurithiosalicylate (EMTS) and platinum diamminodichloride⁴. The X-ray results are summarised in Table 1 and part of the electron density map is shown in Figs 1 and 3.

For metabolite binding studies, crystals were soaked in the standard solution containing 100 mM of the relevant metabolite for 1–2 days. The glucose-1-phosphate solution was found to contain less than 2% inorganic phosphate.

The folding of the polypeptide chain

A total of 801 α -carbon atoms have been placed in the map, leaving 40 atoms unplaced. The amino acid sequence¹¹ has been fitted into two long stretches accounting for over half the molecule and work is now in hand in fitting the remainder. Although these assignments have still to be confirmed by molecular model building, the electron density results for certain key residues support the present interpretation.

Figure 2 shows the structure of phosphorylase *b*. Briefly, it may be described in terms of three domains: (1) the N-terminal domain which contains residues 1–310; (2) the glycogen binding domain with approximately 160 residues; (3) the C-terminal domain with approximately 360 residues. The overall shape of the subunit is compact apart from the two loops (termed the cap, residues 36–45, and tower, residues 244–273, in Fig. 2) which cross the twofold axis at $z = \frac{1}{2}$ and extend into the symmetry-related subunit of the physiologically active dimer. The cap region makes contact with the nucleotide

Table 1 Summary of data and refinement parameters

Derivative	Total no. of terms measured	No. of unique terms	Merging R	Fractional isomorphous difference
Native	81,536	19,480	0.102	
EMTS	80,432	36,641	0.098	0.162
Pt(NH ₃) ₂ Cl ₂	72,638	33,518	0.118	0.142
G1P	68,460	18,159	0.083	0.110
G3	66,287	18,271	0.087	0.103

Derivative	Site	O	x	y	z	B	r.m.s. E	r.m.s. f_H	No. of reflections	R_c
EMTS	Hg 1	0.46	0.1943	0.2244	0.0392	32.7	85	118	17,999	0.55
	Hg 2	0.67	0.0288	0.0885	0.3838	21.3				
	Hg 3	0.16	0.1597	0.3796	0.3246	25.0				
Pt(NH ₃) ₂ Cl ₂	Pt 1	0.53	0.1496	0.3634	0.3385	10.0	91	108	18,199	0.66
	Pt 2	0.30	0.1721	0.3589	0.3514	10.0				
	Pt 3	0.18	0.2517	0.3830	0.2530	25.0				

$$\text{Merging } R = \frac{\sum_h \sum_i |\bar{I}(h) - I_i(h)|}{\sum_h \sum_i I_i(h)}$$

where $\bar{I}(h)$ is the mean intensity of reflection h . Friedel pairs of reflections were not merged for the heavy atom derivatives EMTS and Pt(NH₃)₂Cl₂. O is relative occupancy, E is the r.m.s. lack of closure error, f_H is the r.m.s. calculated structure factor.

$$R_c = \frac{\sum |F_{PH} - |F_P + f_H||}{\sum |F_{PH} - F_P|} \text{ for centric data}$$

The overall figure of merit is 0.54.

binding site on the symmetry-related subunit, and may be important in the control properties of the enzymes. The tower extends approximately 16 Å into the other subunit and there are extensive interactions between the two symmetry-related towers.

From the fold of the molecule it seemed that the most likely binding sites for the mercury atoms Hg-1 and Hg-2 of the heavy atom derivative EMTS were the cysteine residues Cys 782 and Cys 108, respectively. This assignment provides some unexpected results. Cys 782, for example, has not previously been reported as a reactive sulphhydryl although, in the crystal structure its position is relatively exposed (Fig. 2). Cys 108 may now be assigned from the sequence as one of the two slow-reacting sulphhydryl groups which have been identified in solution studies with reagents such as iodoacetamide and whose alkylation is prevented by the presence of AMP¹². In phosphorylase *b* Cys 108 is approximately 27 Å from the AMP binding site but it is in a somewhat buried environment which might well be influenced by allosteric activators. Solution studies have identified two fast-reacting sulphhydryl groups whose reaction does not affect enzyme activity¹³. From knowledge of the complete amino acid sequence these cysteines may be identified as Cys 171 and Cys 317. In the electron density map of phosphorylase *b*, both these residues occur in regions where the path of the polypeptide chain is ill-defined, possibly indicating some flexibility. The minor mercury binding site Hg-3 and the major platinum site occur close to Cys 171. Previous treatment of the crystals with 10 mM iodoacetamide for 5 h before soaking in 1 mM EMTS leads to protection of the Hg-3 site, but no change in the Hg-1 and Hg-2 occupancies. This result, which suggests that Cys 782 and Cys 108 are not readily modified by iodoacetamide, agrees with solution studies and supports the assignment of Hg-3 to Cys 171.

The N-terminal domain contains the two long α -helices, labelled *A-A'* and *B-B'* in Figs 1 and 2, which flank the nucleotide binding site. These two helices seem to be in the same positions as those in phosphorylase *a* (ref. 7). Likewise, comparisons of the published diagrams for phosphorylase *a* and our present results with phosphorylase *b* show similar

positions for the arginine residue of the *B-B'* helix (probably Arg 309) whose reaction with 2,3-butanedione leads to inactivation of the enzyme¹⁴, and the residue on the *A-A'*

Fig. 1 Sections $z = 50/128-57/128$ of the 3-Å electron density map for phosphorylase *b*. The two α -helices that line the nucleotide binding site are labelled *A-A'* and *B-B'*. Arrow 1 indicates the essential arginine (probably Arg 309) from the *B-B'* helix¹⁶ which makes contact with the phosphate of glucose-1-phosphate bound at this site. Arrow 2 indicates the residue (probably Arg 69) which in phosphorylase *a* (ref. 7) makes contact with the phosphate on Ser 14. In phosphorylase *b* this residue is exposed to the solvent. The side chain of a tryptophan residue two residues away from Arg 69 on the inside of the *A-A'* helix is visible.

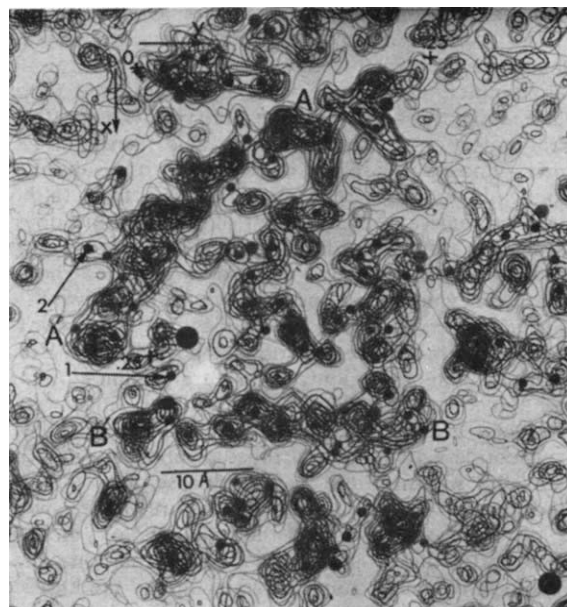
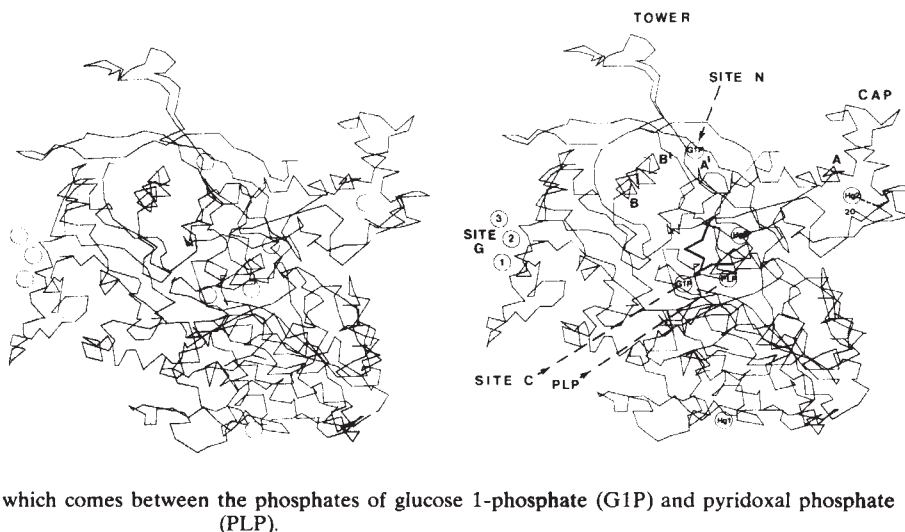


Fig. 2 Stereo diagram of the α -carbon positions of glycogen phosphorylase *b*. Hg-1, Hg-2, Hg-3 mark the three binding sites for the mercury atoms of the heavy atom derivative EMTS which are at cysteine residues 782, 108 and (probably) 171, respectively (see text). The diagram also shows the phosphate binding positions for glucose-1-phosphate, and for pyridoxal phosphate and the three sugar binding sites for maltotriose. The start of the chain at Gly 20 is marked. The dotted region between the end of the *B-B'* helix and the start of the glycogen binding domain is of ill-defined density. The darkened loop of the chain in the right-hand image represents the loop 130-137 which comes between the phosphates of glucose 1-phosphate (G1P) and pyridoxal phosphate (PLP).



helix, identified in our structure as Arg 69, which, in phosphorylase *a*, interacts with the phosphate group on Ser 14. The most obvious difference between phosphorylases *a* and *b* concerns the first 19 residues from the amino terminus which cannot be located in the electron density map for phosphorylase *b*, although there is certainly room for them in the solvent region. This is the region where a large change was observed in the difference Fourier synthesis calculated at 6 Å resolution between phosphorylases *a* and *b* (ref. 6).

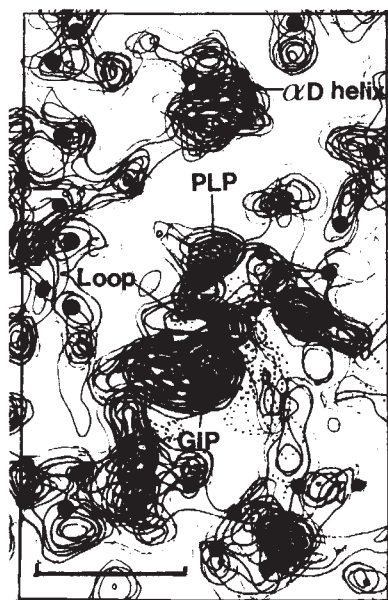
The structure contains two extensive β sheets. One region which extends through the N-terminal domain and the glycogen binding domain has the topology $1x, 3x, -1, -1, -3x, -1x, -1x$ in the notation of Richardson¹⁵. The second region is at the centre of the C-terminal domain and comprises six parallel β strands characteristic of the nucleotide binding domain found in lactate dehydrogenase and other proteins¹⁶. Such a structure was anticipated by Stellwagen *et al.*¹⁷ and first identified in phosphorylase *a* (ref. 7). The sheet region in our map proved one of the more difficult regions to interpret. There are some differences between phosphorylase and lactate dehydrogenase which may be important for the catalytic function of phosphorylase. In phosphorylase the loop between βD and αE and the αE helix (only 1 turn) are shorter and the loop between βD and αC is longer than in lactate dehydrogenase.

Pyridoxal phosphate

Pyridoxal phosphate (PLP) is present in all glycogen phosphorylases studied so far and is essential for activity (see ref. 2 for review). Its role, whether structural or catalytic, has long remained a mystery, but current evidence¹⁸⁻²⁰ favours a catalytic role for the phosphate group. Pyridoxal phosphate at acid pH is bound as a Schiff base to the ϵ -amino group of a lysine residue (Lys 679 from the sequence¹¹) and, in contrast to other vitamin B₆ enzymes, phosphorylase remains active when the Schiff base is reduced by sodium borohydride²¹. Lys 679 and the additional electron density representing the cofactor have been identified using the amino acid sequence and Cys 782 as a marker. Lys 679 is located in the 'nucleotide binding domain' close to the short αE helix. The location of the coenzyme is consistent with several results from solution studies: it is in the interior of the molecule in a hydrophobic region as suggested by fluorescence studies^{22,23}. It is accessible to the solvent through a narrow channel and is likely to be made less accessible on binding glucose-1-phosphate in the presence of nucleotide activator in agreement with the work of Honikel and Madsen²⁴. Recent nuclear magnetic resonance studies

have shown that the phosphate group of pyridoxal phosphate does not become exposed on dissociation of the enzyme¹⁹. In the crystal structure the coenzyme is well removed from subunit-subunit contacts. Shortly after this assignment of the pyridoxal phosphate site had been made, we learned that a similar position for the coenzyme had also been located in phosphorylase *a* (ref. 8).

Fig. 3 Sections $z = 28/128-34/128$ of the 3-Å electron density map of phosphorylase *b* with the difference Fourier synthesis for glucose-1-phosphate superimposed. Negative contours in the difference synthesis are shown dotted. The labels PLP and G1P point to the phosphate positions assigned to the electron density for the pyridoxal phosphate and glucose-1-phosphate, respectively. The loop of chain from the N-terminal domain which separates the two phosphates is visible and is marked by two black dots. There are indications of small conformational changes in this region. The short αD helix of the nucleotide binding domain is at the top of the figure. Part of a helix from the glycogen binding domain is visible in the lower left with an aromatic residue (possibly a tryptophan) making contact with the sugar moiety of the glucose-1-phosphate. The electron density in the lower right corner corresponds to the loop which links the tower to the *B-B'* helix. The bar corresponds to 10 Å.



Metabolite binding sites

Low resolution binding studies¹⁰ with a variety of metabolites to phosphorylase *b* have led to the identification of three major binding sites on the molecule: (1) a site in the N-terminal domain (termed site *N*) which binds the allosteric nucleotides AMP, ATP and the substrates glucose-1-phosphate and arsenate and the inhibitor glucose-6-phosphate; (2) a glycogen storage site (termed site *G*) in the glycogen binding domain which binds maltotriose; (3) a second glucose-1-phosphate binding site (previously termed G1P(2) and now termed site *C*) situated in the C-terminal domain but close to the region where the three domains come together and to the pyridoxal phosphate site (Figs 2–4). Undoubtedly, these three sites are an oversimplification and more sites are likely to be identified in further studies.

At 3 Å resolution, glucose-1-phosphate binds twice as strongly to site *C* as to site *N*. The structure of α -glucose-1-phosphate²⁵ has been fitted to the difference electron density using a computer display system²⁶ (Fig. 3). There are small local conformational changes, including residues from the bottom of the tower and the 130–137 loop, but there does not seem to be any substantial rearrangement of the molecule.

Glucose-1-phosphate also binds weakly at site *N* with a peak height approximately 0.42 times that at the major binding site. The phosphate moiety of glucose-1-phosphate makes contact with the essential arginine on the *B*–*B'* helix while the sugar is in contact with the cap region of the symmetry-related subunit and with residues on the *A*–*A'* helix (including Trp 67). The separation of site *N* from site *C* is 33 Å (phosphate-to-phosphate distance) through the molecule.

At 3 Å resolution maltotriose binds at the glycogen storage site (site *G*) on the surface of the molecule in the glycogen binding domain, and makes contact with part of the long α -helix and one of the β strands of that domain. The three glucose rings are readily identified in the difference electron density which exhibits a definite curve characteristic of the helical twist of amylose²⁷. There are no indications of conformational changes except for some local movement close to the maltotriose site. The closest distance between this site (that

Fig. 4 A schematic diagram of the phosphorylase *b* dimer viewed down the molecular twofold axis showing the domains and the binding sites for glucose-1-phosphate and maltotriose as determined from 3-Å studies.

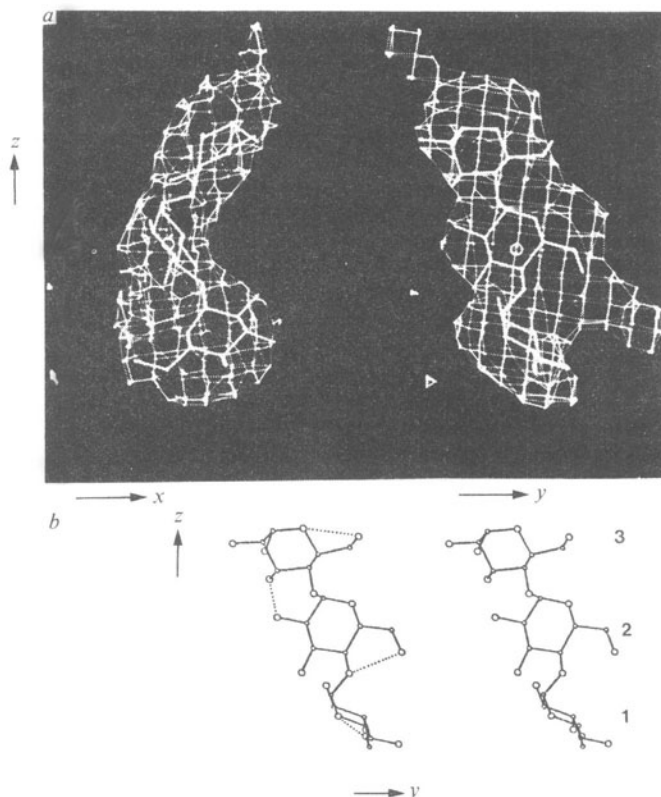
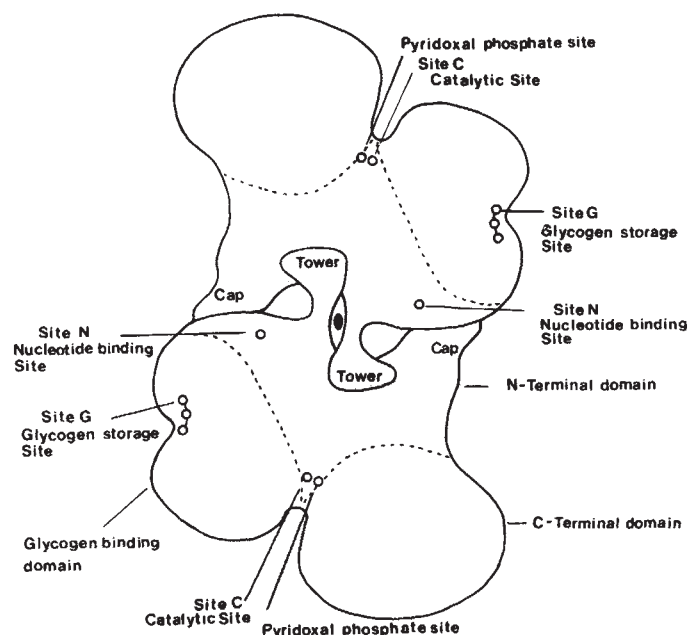


Fig. 5 *a*, The difference electron density for maltotriose at the glycogen storage site, site *G*, and the fit of the molecule obtained from the computer controlled CRT display. Two orthogonal views are displayed. The reducing end of maltotriose is at the top in this figure and in Fig. 2. *b*, A stereo view in the *yz* plane of the maltotriose molecule. The dotted lines indicate possible hydrogen bonds.

is, the O4 of glucose 1) and site *C* is 33 Å and the closest distance (that is, O1 atom of glucose 3) to the moiety of glucose-1-phosphate at site *N* is 40 Å. The difference electron density for maltotriose has been fitted on the cathode ray tube (CRT) display using as starting coordinates three glucose residues from the crystal structure of cyclohexaamylose²⁸ (Fig. 5). Analysis of dihedral angles of the α (1–4) glycosidic linkages showed that the relative conformation of sugars 3 and 2 is close to that observed for cyclohexaamylose²⁸ and to the major minimum energy conformation obtained from theoretical studies²⁹. In this conformation an intramolecular hydrogen bond is formed between O2(2) and O3(3), where the number in parentheses refers to the sugar residue. It was apparent from the electron density map that sugars 1 and 2 had a different relative conformation and the structure finally obtained from the CRT fitting is found to be close to the subsidiary minimum energy conformation described by Rees^{29,30}. These results suggest that in amylose there is a certain amount of conformational flexibility about the α (1–4) linkage which can be used by the protein to provide a tight binding site.

The next most significant feature in the maltotriose difference Fourier synthesis is at the N-terminal domain site where maltotriose was observed to bind in the low resolution studies¹⁰. However, at 3 Å the peak is only half as strong as the major binding site for maltotriose and is too small in volume to represent three glucose molecules. The site is approximately 4 Å from the glucose binding site of glucose-1-phosphate in this region. The reason for the difference between the high resolution result and the earlier studies¹⁰ (where the ratio between the N-terminal domain peak and the glycogen storage peak was 0.8:1) is not clear. It is possible that this peak represents a tightening of the binding of IMP at the allosteric nucleotide site, but this remains to be established.

Discussion

At the present stage in the analysis, no detailed comparison of phosphorylases *a* and *b* has been carried out. However, it is apparent that a major difference exists in the N-terminal region of the chain. In phosphorylase *b*, the first 19 amino acids have not been located in the electron density map and thus seem to have considerable mobility. In phosphorylase *a* these residues (apart from the first eight residues from the N-terminus) are apparently well ordered and the phosphate group attached to Ser 14 interacts with a basic group, probably Arg 69 on the A-A' helix. The flexibility of these residues in phosphorylase *b* is compatible with the results of McCarty *et al.*³¹ who have shown that a 14-residue oligopeptide (corresponding to residues 5–18) containing a phosphorylated Ser 14 induces phosphorylase *a* properties in phosphorylase *b*. Presumably, the flexible nature of the N-terminal portion of phosphorylase *b* allows the binding of the phosphorylated peptide.

Flexible regions have been observed in other proteins and have sometimes been implicated in the conversion of the enzyme from an inactive to an active species (see ref. 32 and references therein). However, the extent to which the localisation of the first 19 residues in phosphorylase is correlated with the active structure of the enzyme remains to be seen, although these residues are undoubtedly important in the control properties. For example, solution studies have shown that phosphorylase *b'*, a tryptic derivative of phosphorylase *a* which lacks the first 16 residues, is active in the presence of AMP but devoid of homotropic and heterotropic cooperative effects^{33,34}.

The location of the active site in phosphorylase has not been straightforward. Early work^{7,10} had suggested that the N-terminal domain binding site (site *N*) was the active site of phosphorylase; the proximity of the site to the subunit-subunit interface could readily account both for the allosteric behaviour of the enzyme and for the observation that monomers of phosphorylase are inactive³⁵. However, the present results show that glucose-1-phosphate binds twice as tightly to site *C* as to site *N* and that site *C* is close to the pyridoxal phosphate. There are significant differences between glucose-1-phosphate binding at site *C* to phosphorylases *b* and *a*. In phosphorylase *b* glucose-1-phosphate binds tightly at 100 mM with only small local conformational changes. In phosphorylase *a*, glucose-1-phosphate only binds at high (300 mM) concentrations and produces large changes in structure remote from the catalytic site⁸.

In view of the accumulating evidence^{18–20} that the phosphate group of the pyridoxal phosphate is involved in catalysis, it seems reasonable to assign the active site to site *C*. The phosphate of the coenzyme is close enough to the substrate for it to be involved in catalysis, although probably not directly so as there is a loop of chain intervening between the two phosphates. The sequence of this loop in phosphorylase (Gly-Leu-Gly-Asn-Gly-Gly-Leu-Gly) has a similar pattern of alternating glycine residues to the sequence 16–22 in adenylate kinase (Gly-Pro-Gly-Ser-Gly-Lys-Gly). In the active conformation of adenylate kinase³⁶ the 'glycine' loop closes the entrance to the AMP binding pocket wrapping around the phosphate ion and forms the epicentre for the structural changes between the active and the inactive conformation. In phosphorylase *b* it is tempting to speculate that this region of high glycine content might have a similar role. The new catalytic site has the advantage that it is buried at the bottom of a deep crevasse in an environment which in the ternary complex is likely to exclude water and thus to favour phosphorylysis rather than hydrolysis.

At its narrowest point the crevasse has dimensions 6 × 8 Å and is just wide enough to accommodate the second substrate glycogen. However, no binding of the glycogen analogue, maltotriose, has been observed in this region. Instead, both in our studies and in those on phosphorylase *a* (ref. 37) maltotriose binds to a site on the surface of the molecule some 33 Å from site *C* and 40 Å from site *N*. Kinetic studies have shown

that phosphorylase activity can be enhanced by preincubation with glycogen and the results interpreted in terms of a glycogen binding site distinct from the catalytic site³⁸. This site seems to be important physiologically, for it is possible to isolate from muscle a well defined structural entity rich in glycogen to which are attached phosphorylase and other enzymes of glycogen metabolism in a protein glycogen complex³⁹. Kinetic studies with phosphorylase *a* have suggested that this site needs to be filled before glycogen can bind at the catalytic site³⁷.

In this interpretation, the N-terminal domain binding site is assigned as a nucleotide binding site. It is encouraging to find that Tyr 155, the tyrosine residue which can be covalently modified by an analogue of AMP^{40,41} is in the vicinity of this site. There is no obvious way in which the site communicates with the active site, although the loop of chain involving residues 130–137 and the loop which connects the tower to the B-B' helix are possible regions through which conformational changes from one site could be relayed to the other site.

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Structure and processing of yeast precursor tRNAs containing intervening sequences

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We have isolated a precursor of yeast tRNA^{Tyr} and shown that it contains an intervening sequence identical to that found in the gene for tRNA^{Tyr}. The conformation of pre-tRNA^{Tyr} is similar to that of mature tRNA^{Tyr} except for the anticodon loop. The loop is sensitive to endonucleolytic cleavage by S₁ nuclease near to the ends of the intervening sequence. This pre-tRNA is functionally inactive as it cannot be aminoacylated and the anticodon is not accessible for hydrogen bonding. A crude nuclear extract from yeast contains an excision-ligase activity which will process pre-tRNA^{Tyr} into mature tRNA^{Tyr}.

ANALYSIS of the genes coding for mouse¹ and rabbit globin², ovalbumin³ and immunoglobulin⁴ have resulted in the unexpected finding that these DNAs contain additional sequences which are not used in encoding the final gene product. Further evidence that genes are not necessarily co-linear with their gene products derives from experiments showing that several viral mRNAs contain sequences complementary to non-adjacent regions of their genomes⁵⁻⁹. We have also previously shown that, immediately to the 3' side of their anticodon triplets, the yeast genes coding for tyrosine and phenylalanine tRNAs^{10,11} contain a base pair tract, called an intervening sequence, that is not present in the mature tRNA.

There are eight unlinked genetic loci for tRNA^{Tyr} in yeast¹² and the nucleotide sequence of three have been determined¹⁰. One of these was identified and analysed as coding for the suppressor tRNA associated with the genetic locus *SUP* 4 (ref. 10). All three genes contained a 14 base pair intervening sequence, ATTTAYCACTACGA (Y = pyrimidine). Two genes (called G and C in plasmid pYT-G and pYT-C, respectively) had a C, and one gene (called A in plasmid pYT-A) had a T at the position indicated by Y. Analysis of the *SUP* 4 locus for tRNA^{Tyr} demonstrated that the intervening sequence was present in an 'active' gene, as this mutation confers a dominant phenotype on *SUP* 4-0 strains and the mutant gene contained the intervening sequence¹⁰.

There are at least 10 unlinked tRNA^{Phe} genes in a tetraploid yeast strain¹³. The nucleotide sequence of four alleles has been determined to contain an intervening 18 or 19 base pair sequence, AAAAAGCTTCGGTCAAGTTA or AATACTTCGGTCAAGTTA (ref. 11). Thus, the intervening sequences in these two different tRNA genes have no obvious sequence homology or structure, yet both occur in a similar position in the tRNAs.

These findings indicate that the biosynthetic pathway for some if not all tRNAs and mRNAs must involve elimination of the intervening sequence. Various possibilities for the removal of the intervening sequence include DNA splicing, RNA polymerase jumping, or cleavage and ligation of RNA precursors. We report here evidence for the latter mechanism, as

direct nucleotide sequence analysis indicates that one isolated RNA precursor to yeast tRNA^{Tyr} contains the intervening sequence. Using crude nuclei preparations from yeast, pre-tRNA^{Tyr} and several other precursor tRNAs have been processed into tRNA^{Tyr} and 4S size molecules, respectively. Evidence is presented that pre-tRNA^{Tyr} has an overall conformation similar to that of mature tRNA^{Tyr}, except in the anticodon stem. In contrast to mature tRNA, it cannot be aminoacylated. Evidence has been obtained by Knapp *et al.*¹⁴ for the presence of intervening sequences in several yeast tRNA precursors and for their removal *in vitro* by yeast extracts. The existence of such evidence was communicated to us by John Abelson during our own investigations. Our results confirm and extend their observations.

Isolation of a precursor to tRNA^{Tyr} which contains the intervening sequence

The presence of intervening sequences in yeast tRNA genes suggested that yeast tRNA may be derived from a precursor containing the intervening sequence. Evidence has already been obtained for the transcription of intervening sequences in the case of the 15S precursor to globin mRNA (ref. 15).

To determine conditions which maximise synthesis of precursor RNAs we compared the amount of 4.5S ³²P-labelled RNA synthesised in a 15-min pulse at 37 °C in either wild-type yeast, mutant *ts*136 (refs 16, 17) which accumulates larger size RNAs at 37 °C (ref. 18, 47, and A. Hopper and F. Banks, personal communication), or in a cell-free extract of nuclei prepared from *ts*136 cells. The RNA prepared from mutant *ts*136 cells labelled *in vivo* synthesised the largest amount of 4.5S RNA (data not shown). We demonstrated that this extract contained a precursor for tRNA^{Tyr} by hybridisation to the DNA insert from clone pYT-G which contains the gene for tRNA^{Tyr} in a 1.2 kilobase yeast *Eco*RI DNA fragment¹⁰. The hybridised RNA was isolated by chromatography on Agarose 5M (data not shown; RNA isolated as in ref. 19). RNA sequence analysis²⁰ of the hybridised RNA indicated that it was a precursor of tRNA^{Tyr}. This method, however, was not applicable to isolation and preparative quantities of pre-tRNA^{Tyr}.

Preparative separation and isolation of pre-tRNA^{Tyr} was achieved by polyacrylamide gel electrophoresis (Fig. 1). Comparison of the profile of separation of RNA isolated from wild type (Fig. 1a) and *ts*136 (Fig. 1b) yeast strains shows a significant increase in the number of species in the region of the gel between 5S RNA and tRNA in the mutant strain. Four of these species have been identified as precursors of tRNAs for tyrosine, phenylalanine, tryptophan and serine (see also ref. 14). The tyrosine and phenylalanine precursors were identified by hybridisation of the RNA eluted from the gel to filters containing cloned DNA specific for the tRNA^{Tyr} or tRNA^{Phe}.

genes, respectively. The tryptophan and serine precursors were identified by direct RNA sequence analysis (ref. 14 and M. J. Etcheverry, D. S. Colby and C. Guthrie, personal communication). As pre-tRNA^{Tyr} occurs in high yield and migrates in a relatively uncrowded region of the gel, it can be isolated in high purity by extraction from the 10% first dimension gel (right hand panel, Fig. 1b).

Pre-tRNA^{Tyr} isolated from the gel was further characterised by T₁ ribonuclease digestion and two-dimensional oligonucleotide separation (Fig. 2). The separation method used was electrophoresis on cellulose acetate paper at pH 3.5 in the first dimension and homochromatography on DEAE, thin layer chromatography plates, in the second dimension^{20,21}. The partial sequence of each of the 18 T₁ oligonucleotides was determined after scraping and elution from the chromatography plates, by subsequent digestion with pancreatic RNase (RNase A) and separation on DEAE paper at pH 3.5 (ref. 22). A summary of these results is presented in Table 1. The sequence of oligonucleotides nos 1, 2, 3, 5, 6, 7, 8, 11 and 13 are uniquely determined from their RNase A digestion prod-

ucts. These sequences were confirmed and the remaining T₁ oligonucleotides identified by comparison of their positions on the fingerprint and their RNase A digestion products with the known sequences of tRNA^{Tyr} (ref. 23) and/or its gene¹⁰. Additional support for these assignments was obtained by the relative migration of the T₁ products on cellulose acetate followed by DEAE paper in 7% formic acid in the second dimension (data not shown; compare with ref. 24), and by localisation of each T₁ product to the 5' or 3' 'halves' of the molecule after S₁ nuclease digestion (see below, Fig. 7). The position of each T₁ product in the sequence is shown in Fig. 3.

Three oligonucleotides (15, 16, 17) occur in the precursor which are not present in the mature tRNA. These must arise from the intervening sequence,

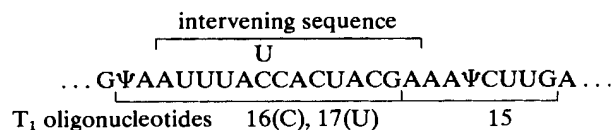
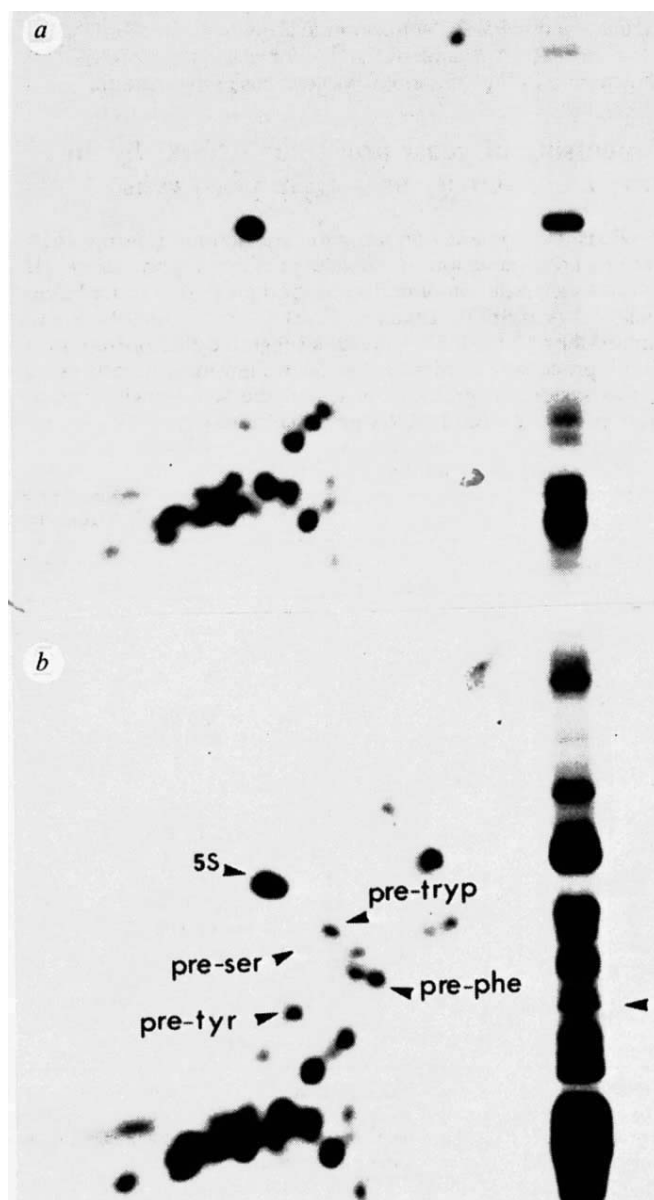


Fig. 1 Two-dimensional electrophoresis of low molecular weight ³²P-labelled RNA from wild-type and *ts136* yeast. Either wild-type A364A or *ts136* yeast cells¹⁶ were grown at 25°C to an optical density of 2.0 at 650 nm in YEPD medium³⁵, collected by centrifugation, washed in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and resuspended in phosphate depleted YEPD medium³⁶. The cells were grown at 25°C for an additional 2 h, collected and resuspended in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 2% glucose. Carrier-free ³²P-phosphate was added (at room temperature) to a concentration of 1 mCi ml⁻¹ and the cells were immediately shifted to 37°C. After a 15-min labelling period, the cells were chilled to 5°C and centrifuged. The radioactive cell pellet was resuspended in one volume 100 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5% sodium dodecyl sulphate (10 ml for a pellet derived from 50 ml of cell culture), one volume phenol saturated with 10 mM Tris-HCl, pH 7.4, and one volume chloroform. The mixture was placed at 68°C with shaking for 15 min. The suspension was centrifuged, the aqueous phase removed, NaCl added to 0.3 M and the RNA precipitated with ethanol at -20°C. The nucleic acid pellet was resuspended in 10 mM Tris-HCl, pH 7.4, 10 mM MgCl₂ and DNase (Worthington, and further purified as in ref. 37) was added to 20 µg ml⁻¹ for 30 min at 37°C. After phenol extraction and precipitation by ethanol, the RNA pellet was resuspended in sample buffer for electrophoresis containing 4 M urea, 50 mM NaCl, 5 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 15% sucrose, 0.1% bromphenol blue and 0.1% xylene cyanol. Two-dimensional electrophoresis was carried out by a modification of the procedure of Fradin *et al.*²⁷. The first dimension was a 10% acrylamide (19:1, acrylamide:bisacrylamide) separating gel 30 cm high containing 4 M urea in Tris-borate buffer, pH 8.3 (ref. 38) and a 5-cm high spacer gel containing 5% acrylamide, 4 M urea in Tris-borate buffer titrated with HCl to pH 6.8. Electrophoresis was for 19 h at 250 V. For the second dimension a strip of gel containing RNAs from 4S to 5.8S was embedded in a 20% acrylamide gel containing 4 M urea + Tris-borate buffer, pH 8.3, and electrophoresed for 3-4 d at 300 V with several changes of running buffer (Tris-borate, pH 8.3). The separation of ³²P-labelled low molecular weight RNA is shown for wild-type (a) and *ts136* (b) cells. The separation achieved in the first dimension (10% gel) is shown on the right. Pre-tRNA^{Tyr} is indicated by an arrow in b. The second dimension is from right to left. Transfer RNA precursors of tRNA^{Tyr} and tRNA^{Phe} were identified by hybridisation of radioactively labelled RNAs to specific DNA probes for either tRNA^{Tyr} (pYT-G, ref. 10) or tRNA^{Phe} (ref. 11). DNA was immobilised onto membrane filters by the procedure of Landy *et al.*³⁹ (2 µg DNA per 5-mm diameter filter). Hybridisation was carried out in a volume of 100 µl containing 3×SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate), 50% formamide, 10 mM HEPES, pH 7.0, and 1X Denhardt solution⁴⁰ at 37°C for 24 h. The filters were washed three times at 37°C with 2×SSC containing 0.5% SDS and the amount of ³²P-RNA hybridised was determined by Cerenkov counting. Pre-tRNA^{Ser} and pre-tRNA^{Tryp} were identified by direct sequence analysis carried out by Etcheverry, Colby and Guthrie (unpublished results and ref. 14).



Their sequence and position in the molecule proves that the intervening sequence in the gene is transcribed and is contained in the precursor. Sequence analysis of the tyrosine genes shows that the intervening sequences differ by a single base change (ref. 10, and see Fig. 3). This heterogeneity is consistent with the sequence of the precursor tRNA^{Tyr}, T₁ products 16 and 17 reflect the sequence change from a C to a U, respectively. The quantitation of these two oligonucleotides (Table 1) suggests that there are three genes with a 'C' in the intervening sequence and five genes with a 'T' (U in the RNA) if all eight tRNA genes have intervening sequences and all are transcribed with equal efficiency.

Although analysis of the minor bases occurring in pre-tRNA^{Tyr} is incomplete (Table 1), evidence has been obtained for the following: dihydrouridylic acid (D) in T₁ oligonucleotides nos 9, 11 and 13; ribothymidylic acid (T) in no. 12; pseudouridylic acid (Ψ) in nos 12, 15, 16 and 17; and m¹A in 4. No 2'-O-methyl guanylic acid seems to occur, and hence oligonucleotide no. 11 is isolated as DDGp rather than DDGmGp as predicted from the sequence of tRNA^{Tyr} (refs 23, 24). In general pre-tRNA^{Tyr} is apparently undermethylated.

Although pre-tRNA^{Tyr} is undermodified, both the 5' and 3' ends are already processed: the 5' end does not contain additional nucleotides and the 3' terminal CCA_{OH} has been added. Figure 3 summarises the nucleotide sequences for the tRNA^{Tyr} gene, pre-tRNA^{Tyr} and tRNA^{Tyr}. For clarity, pre-tRNA^{Tyr} is shown (and in Fig. 8b below) without base modification.

Processing of yeast precursor tRNAs by an enzymatic activity present in yeast cells

Previous experiments on *in vitro* transcription using yeast nuclei and endogenous or exogenous RNA polymerase III showed accumulation of molecules of the size of but not larger than tRNA (4S) (P. Tekamp, G. Bell, P. V. and W. J. R., unpublished results). These results suggested that presumptive tRNA precursors of high molecular weight must be processed in this system. Therefore, we tested the same nuclear preparations as a source of tRNA processing activity.

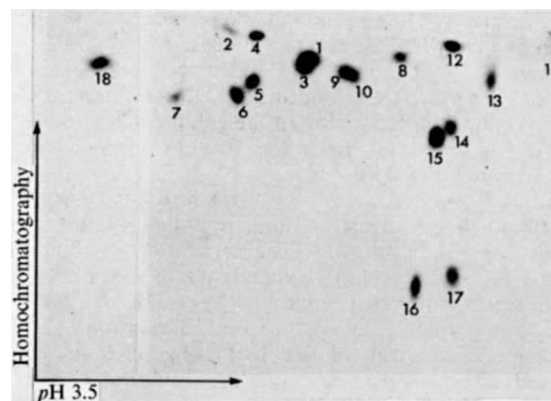


Fig. 2 T₁ RNase oligonucleotides from pre-tRNA^{Tyr}. The ³²P-labelled band corresponding to pre-tRNA^{Tyr} was cut from a 10% gel and eluted by first grinding the dry gel into small pieces followed by addition of 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.3 M NaCl. The gel pieces were eluted overnight at 0°C with shaking. The eluate was filtered through a nitrocellulose Millex filter (0.45 μm) and precipitated with ethanol. The RNA was hydrolysed by RNase T₁ (Calbiochem) and the resulting oligonucleotides were separated in two dimensions by electrophoresis on cellulose acetate at pH 3.5 (ref. 20), and homochromatography on DEAE cellulose at 60°C using homomix B (ref. 21). The oligonucleotides are numbered 1–18 and are described in Table 1. As oligonucleotide 18 moved much more slowly than the other products in the first dimension, it was chromatographed on a separate homochromatography plate. Pre-tRNA^{Tyr} used for the analysis was prepared as in Fig. 1 except that the cells were labelled to low specific activity as described in Table 2.

Eight different pre-tRNAs were processed *in vitro* to 4S molecules using extracts of nuclei prepared from wild-type yeast. Figure 4 shows the results using five different pre-tRNAs. The preparations of nuclei are reasonably free of nonspecific nucleases, as no degradation of 5S RNA occurs (Fig. 4, lanes 2 and 3).

Table 1 T₁ oligonucleotides of precursor tRNA^{Tyr}

Oligo-nucleotide no.	Molar yield Observed	Theoretical	Pancreatic RNase products	Minor bases	S ₁ nuclease fragment	Sequence
1	6.6	7	G		5':3', 3:4	Gp
2	2.1	2	C, G		5':3', 1:1	CGp
3	3.2	2	AG		3'	AGp
4	0.54	1	m ¹ AC, C, U, G	m ¹ A	3'	m ¹ ACUCGp
5	1.5	1	C, AAG		5'	CAAGp
6	1.3	1	C, AAG		5'	CCAAGp
7	0.47	1	C, G		3'	CCCCCGp
8	1.4	1	AG, U		5'	UAGp
9	0.96	1	AD, G, C	D	3'	ADCGp
10	1.1	1	AC, G, U		5'	ACUGp
11	1.5	1	DD, G	D	5'	DDGp
12	1.5	1	G, C, T, Ψ	T, Ψ	3'	TΨCGp
13	0.95	1	AAG, DD, D	D	5'	DDDAAGp
14	0.71	1	pCp, G, C, U		5'	pCUCUCGp
15	1.1	1	AAAΨ, G, C, U	Ψ	5'	AAAΨCUUGp
16	0.37	1	AAU, AC, C, G, U, Ψ	Ψ	—	ΨAAUUUACACUACGp
17	0.57	1	AAU, AU, AC, C, G, U, Ψ	Ψ	—	ΨAAUUUAUCACUACGp
18	0.95	1	AC, C		3'	ACCA _{OH}

The individual oligonucleotides visualised in Fig. 2 were recovered from the homochromatography plate and counted in scrapers by Cerenkov radiation. The molar yields were calculated from the counts incorporated into each oligonucleotide relative to the average number of counts incorporated per phosphate. The composition of each oligonucleotide was determined by hydrolysis with RNase A and subsequent electrophoresis at pH 3.5 on DE81 paper²². Analysis of minor bases was carried out by T₂ ribonuclease digestion of the RNase A products and two-dimensional thin layer chromatography^{33,34}. Oligonucleotides were ascribed to either the 3' or 5' side of pre-tRNA^{Tyr} following treatment of the RNA with S₁ nuclease which cleaves tRNA into 'halves' (see Fig. 7). T₁ products 1 and 2 are found in both S₁ 'halves' and their molar yields in each half are indicated. Although these data provide only partial sequence analysis, the actual sequence of pre-tRNA^{Tyr} has been deduced by comparison with the sequence of tRNA^{Tyr} (refs 23, 24) and the sequence of the tRNA^{Tyr} gene¹⁰.

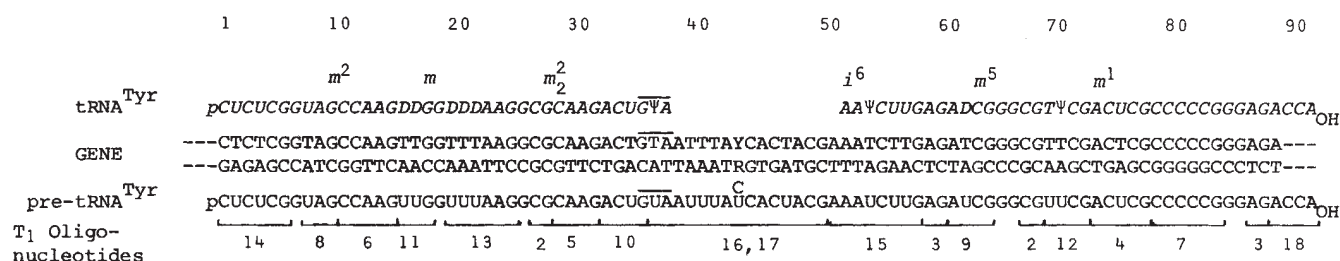
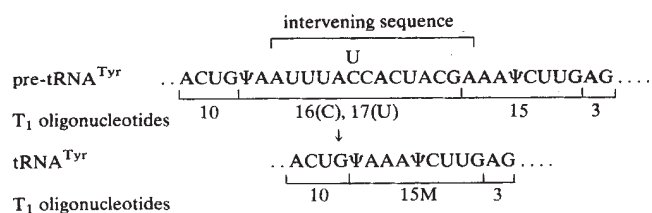


Fig. 3 The nucleotide sequence of yeast tRNA^{Tyr}, the tRNA^{Tyr} gene, and pre-tRNA^{Tyr}. Only the actual coding part of the gene is shown, although the nucleotide sequence on either side has been determined¹⁰. In addition, there is no nucleotide sequence in the gene for the 3' terminal CCA^{OH} (ref. 10). The anticodon and its coding triplet are overlined in both RNA and DNA sequences. For simplicity, no minor bases are indicated for pre-tRNA^{Tyr} (see Table 1 for an estimate of their composition). The T₁ oligonucleotides 2–18, shown in Fig. 2 and described in Table 1, are indicated below the linear sequence of pre-tRNA^{Tyr}; T₁ product no. 1 (nucleotide G) is not shown for simplicity. The variable base pairs within the intervening sequence of the gene are indicated by Y (pyrimidine) and R (purine). The position of the intervening sequence displayed is the most reasonable considering the analysis of the conformation of pre-tRNA^{Tyr} in Fig. 7 below.

The processing reaction was optimised for processing activity with respect to NaCl, MgCl₂ and ATP concentrations (Fig. 5). The optimal conditions are 50 mM Tris-HCl (pH 8), 10 mM 2-mercaptoethanol, 8 mM MgCl₂, 160 mM NaCl and 0.8 mM ATP. The reaction is highly dependent on ATP although GTP seems to be able to replace ATP in this crude system. At equivalent concentrations, NaCl may be replaced by KCl, LiCl or NH₄Cl with identical activity. The reaction is inhibited by yeast tRNA.

The processing activity, tRNA excision-ligase, is found in extracts from broken yeast cells. All the enzyme can be recovered in soluble form following centrifugation of extracts at 100,000g in 0.5 M KCl (data not shown).

The mobility of the processed precursor tRNA on a denaturing polyacrylamide gel indicates proper excision of the intervening sequence and rejoining of the ends to form mature tRNA (Fig. 4). The fidelity of the *in vitro* reaction was further characterised using pre-tRNA^{Tyr} as a defined substrate. ³²P-labelled pre-tRNA^{Tyr} was treated with yeast nuclei and the resulting products analysed by electrophoresis (Fig. 4b). T₁ ribonuclease digestion and fingerprint analysis of the 4S RNA shows that it is authentic tRNA^{Tyr} (Fig. 6). All the T₁ oligonucleotides of tRNA^{Tyr} are present. T₁ oligonucleotides 16 and 17 which contain the intervening sequence of the precursor are absent (compare Figs 2 and 6). Oligonucleotide 15 is also absent and is replaced by 15M. The sequence of this T₁ product is ΨAAAΨCUUG (preliminary analysis suggests there is also modified A in this oligonucleotide) and is the critical oligonucleotide proving the precise excision of the intervening sequence and re-ligation to form mature tRNA^{Tyr}. This is schematically shown below.



The predicted intermediates from the *in vitro* processing of pre-tRNA^{Tyr} may also be produced in the *in vitro* reaction. These compounds are visible on the gel as discrete faster migrating bands labelled 'a' and 'b' (Fig. 4b). When band 'a' was recovered from the gel and re-electrophoresed in a 20% acrylamide gel it could be resolved into two species 38 and 40 nucleotides long. Subsequent fingerprint analysis of both fragments confirmed that they are the 3' and 5' 'halves' of the precursor following removal of the intervening sequence (data not shown). Band 'b' was shown by re-electrophoresis on a 20% gel to contain fragments of RNA similar in size to the intervening sequence excised from pre-tRNA^{Tyr}. The possi-

bility that these compounds are selective degradation products of an S-1 like endonuclease activity in the extracts has not been eliminated.

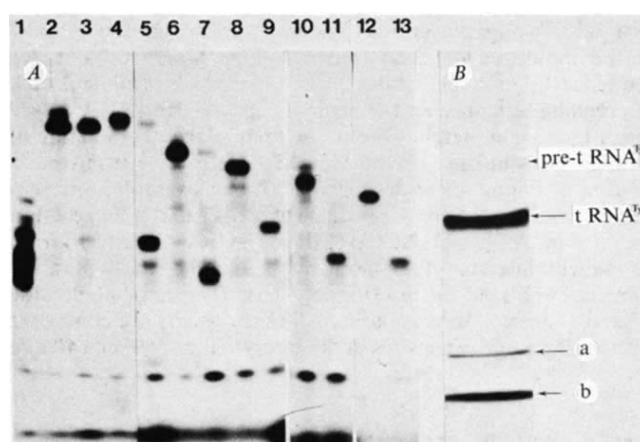


Fig. 4 Processing of yeast tRNA precursors to molecules of 4S size by an enzyme system from yeast nuclei. **A**, Assay of processing activity using several pre-tRNAs and 5S RNA as substrates. Yeast nuclei were isolated from strain B1279 (ref. 41) by the method of May described by Duffus⁴² except that spheroplasts were lysed in a Parr bomb⁴³ at 800 p.s.i. The tRNA precursors used as substrates were isolated from ts136 yeast cells following pulse-labelling with ³²P and gel electrophoresis in a 10% gel as described in the legend of Fig. 1. Each assay tube contained approximately 5 × 10⁴ c.p.m. of RNA, 50 mM Tris-HCl, pH 8, 8 mM MgCl₂, 0.8 mM ATP, 10 mM 2-mercaptoethanol, 160 mM NaCl and 5 μl of yeast nuclei (containing the equivalent of 1 μg DNA). After incubation at 27°C for 25 min, the tubes were centrifuged (3,000g) to remove the nuclei and the supernatant was made 0.1% in SDS. After heating at 65°C for 3 min the tube contents were loaded onto an 11% acrylamide slab gel prepared and run as described by Rubin⁴⁴. 1, Total yeast 4S RNA; 2, 5S RNA; 4, pre-tRNA^{Leu} (J. Abelson, personal communication and ref. 14) 6, unidentified putative pre-tRNA; 8, unidentified putative pre-tRNA; 10, pre-tRNA^{Phe}; 12, pre-tRNA^{Tyr}. Lanes 3, 5, 7, 9, 11 and 13 correspond to the same RNAs as in lanes 2, 4, 6, 8, 10 and 12, respectively, but after incubation with the nuclei system described above. **B**, Preparative processing of pre-tRNA^{Tyr}. ³²P-labelled yeast pre-tRNA^{Tyr} (2 × 10⁶ c.p.m.) was incubated in 0.05 M Tris-HCl, pH 8, 8 mM MgCl₂, 0.8 mM ATP, 10 mM 2-mercaptoethanol, 160 mM NaCl, and 40 μl of yeast nuclei (equivalent to 8 μg of DNA) in a total volume of 0.45 ml. After incubation at 27°C for 30 min, the tube was centrifuged and the supernatant made 0.1% in SDS. After heating for 3 min at 65°C, the sample was loaded in a preparative 11% acrylamide slab and electrophoresed as in **A**. RNA bands were located by autoradiography, excised and eluted as described in the legend of Fig. 1.

Conformation of pre-tRNA^{Tyr}

Susceptibility of an RNA molecule to S_1 nuclease digestion has been a successful method for assessing its conformation^{25,26}. S_1 nuclease cleaves RNA predominantly in accessible single-stranded regions and in all tRNAs studied hydrolyses primarily at the anticodon loop and the 3' single-stranded CCA_{OH} terminus producing oligonucleotides with 5'-phosphate termini.^{25,26}

³²P-labelled pre-tRNA^{Tyr} was digested with S_1 nuclease using limiting conditions such that less than 50% of the molecules were cleaved and purified by gel electrophoresis. The size of the two fragments produced from pre-tRNA^{Tyr} was estimated to be 40–42 nucleotides for the faster migrating fragment and 52–54 nucleotides for the more slowly migrating component (data not shown). The size of the fragments indicates that the site of cleavage of pre-tRNA^{Tyr} is within the intervening sequence.

This conclusion was substantiated by T_1 ribonuclease digestion and fingerprinting of the two fragments obtained after S_1 digestion of pre-tRNA^{Tyr} (Fig. 7). The assignments of oligonucleotides were based on their position in the fingerprint (compare with Fig. 2), RNase A digestion (data not shown) and in certain cases minor base analysis (data not shown).

The fingerprint of the larger fragment (Fig. 7a) is unique in that only T_1 oligonucleotides known to arise from the '3'-side' of the molecule are seen (see also Fig. 3 and Table 1 for sequences). Oligonucleotides 16 and 17, which result from the intervening sequence and occur in uncleaved pre-tRNA^{Tyr} (see Fig. 2), are completely absent. In their place are a series of weaker spots in the area of spot 15. These arise from the 3' portion of oligonucleotides 16 and 17. For example, the relative size and the RNase A products of spots a and b suggest that they are pUACCACUACGp and pUUACCACUACGp from oligonucleotide 16. Other spots in this region of the separation were not analysed but presumably arise from oligonucleotide 17 and contain U in place of C. All these results are consistent with major S_1 cleavage sites in the intervening sequence 40 and

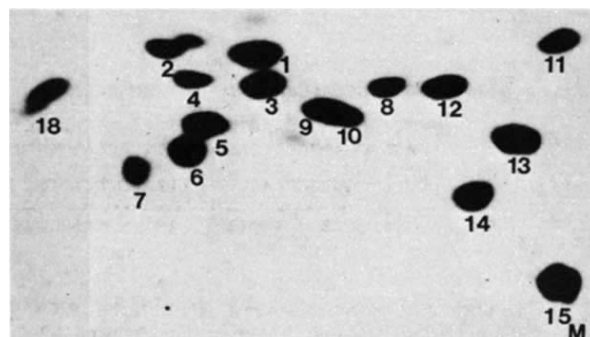


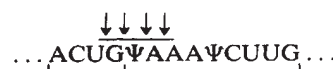
Fig. 6 T_1 RNase oligonucleotides from *in vitro* matured tRNA^{Tyr}. The band migrating in the region of mature tRNA^{Tyr} in Fig. 4b was eluted, digested with T_1 RNase and the resulting oligonucleotides were resolved as described in Fig. 2. The oligonucleotides which are identical to those in Fig. 2 are labelled 1–14. A new oligonucleotide spot corresponding to the anticodon loop of mature tRNA^{Tyr} is labelled 15 M.

41 nucleotides from the 5' end of pre-tRNA^{Tyr}. These cleavages then give rise to a 3' fragment 51 or 52 nucleotides long (Fig. 7a) and a 5' fragment 41 or 40 nucleotides long. (Pre-tRNA^{Tyr} is 92 nucleotides long.)

The fingerprint of the smaller fragment is consistent with its being from the 5' end of the molecule (Fig. 7b). All the T_1 oligonucleotides (nos 1, 2, 5, 6, 8, 10, 11, 13 and 14) from the 5' side are present in close to molar yield. Two new intense spots, e and f (Fig. 7b), also appear. These have been quantitated and analysed by RNase A digestion. They have the sequences UAAUU_{OH} (e; Up (1.16), AAUp (1.0)) and UAAUUU_{OH} (f; Up (2.04), AAUp (1.0)). These must arise by S_1 cleavage of oligonucleotides 16 and 17 at the major sites at positions 40 (produces e) and 41 (produces f) from the 5' end of the precursor.

Also visible in Fig. 7b is a minor set of T_1 oligonucleotides (nos 1, 2, 3, 4, 7, 9, 12, 15 and 18) which occur in about 20% molar yield and all of which arise from the 3' side of the precursor. These data are only consistent with the occurrence of an additional 'minor' (20%) S_1 cleavage site(s) at positions 50 or 51 from the 5' end. This minor cleavage produces a 3' fragment identical in size to the major 5' product.

Pre-tRNA^{Tyr} was processed into mature tRNA^{Tyr} (for example, Fig. 4b). After limited S_1 nuclease cleavage of this RNA the two fragments (35 and 43 nucleotides long) were isolated and digested with T_1 ribonuclease and fingerprinted (Fig. 7c, d). Analysis of the fragments as described above for pre-tRNA^{Tyr} shows that the larger fragments arise from the 3' side of the molecule and the smaller fragment from the 5' portion of the molecule. As the yield of oligonucleotides 10 and 15M are reduced, S_1 cleavage occurs at the anticodon as indicated below:

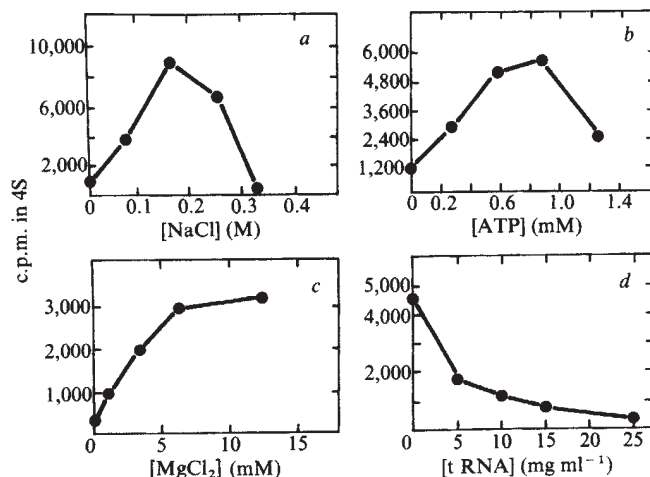


T_1 oligonucleotides 10 15M

(The overlined sequence $\overline{\text{G}\Psi\text{A}}$ is the anticodon. S_1 nuclease cleavage is indicated by the arrows.)

These data are consistent with the secondary structure models of pre-tRNA^{Tyr} and tRNA^{Tyr} shown in Fig. 8. cleavage occurs in accessible single-stranded regions. However, the anticodon is protected in a hydrogen-bonded structure in the precursor (Fig. 8b), but is completely single-stranded in the mature tRNA (Fig. 8a).

Fig. 5 Properties of a yeast enzyme system that removes intervening sequences from pre-tRNAs (excision-ligase activity). Yeast nuclei (containing the equivalent of 1 μ g of DNA) were incubated in 50 mM Tris-HCl, pH 8, 10 mM 2-mercaptoethanol, with $2-5 \times 10^5$ c.p.m. of a mix of yeast ³²P-tRNA precursors (4.5S RNA) for 30 min at 27°C. After electrophoresis and autoradiography, the bands migrating in the 4S region were excised and counted. a, Effect of [NaCl], tubes contained 0.8 mM ATP and 8 mM MgCl₂; b, effect of [ATP], tubes contained 160 mM NaCl and 8 mM MgCl₂; c, effect of [MgCl₂], tubes contained 0.8 mM ATP and 160 mM NaCl; d, inhibition by total yeast tRNA, tubes contained 0.8 mM ATP, 8 mM MgCl₂ and 160 mM NaCl.



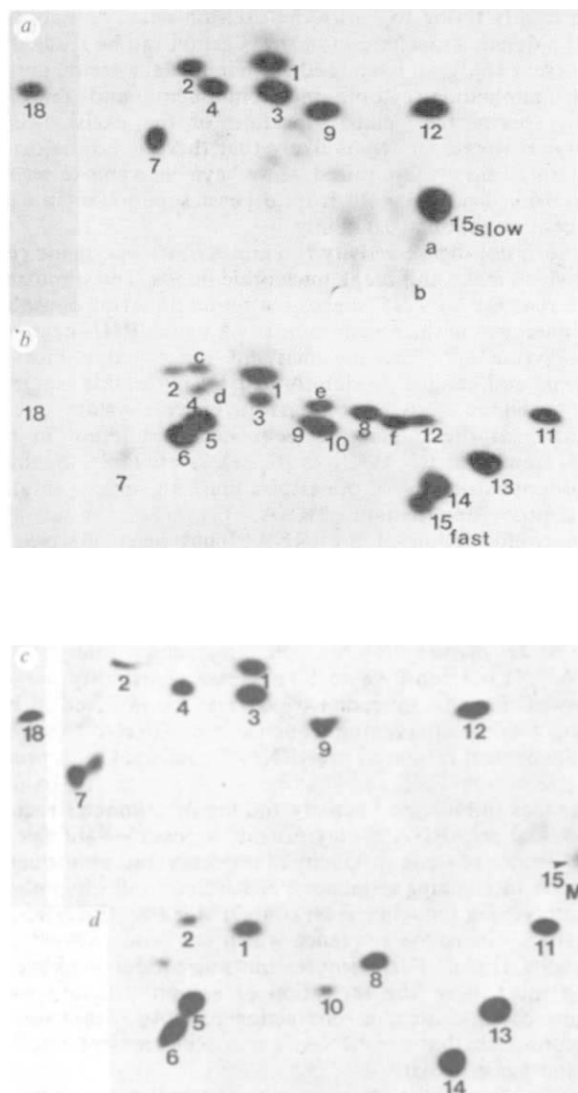


Fig. 7 Analysis of pre-tRNA^{Tyr} and tRNA^{Tyr} treated with S₁ nuclease. S₁ endonuclease was prepared by the method of Vogt⁴⁵ and further purified by filtration through Sephadex G75 according to Ando⁴⁶. [³²P]pre-tRNA^{Tyr} or [³²P]tRNA^{Tyr} was digested with S₁ nuclease at room temperature for 2 h in a reaction mixture containing yeast RNA carrier at 100 µg ml⁻¹, 0.3 M NaCl, 30 mM Na acetate, pH 4.5, 3 mM ZnCl₂. The reaction was limited to 50% digestion of the RNA. RNA was extracted with phenol, precipitated with ethanol and electrophoresed in a 20% acrylamide gel containing 4 M urea and Tris-borate buffer, pH 8.3 (ref. 38). The S₁ cleavage products were located in the gel by autoradiography, excised and eluted as described in legend of Fig. 1. The fragments were hydrolysed with T₁ RNase and the oligonucleotides were separated in two dimensions as in Fig. 2. Individual oligonucleotides were identified by digestion with RNase A and/or RNase T₂ (ref. 22) and are numbered in correspondence with the sequence map presented in Fig. 3 for pre-tRNA^{Tyr} and Fig. 6 for tRNA^{Tyr}. *a*, The T₁ RNase oligonucleotides present in the more slowly migrating or larger S₁ fragment of pre-tRNA^{Tyr}. *b*, The T₁ RNase oligonucleotides present in the faster migrating or smaller S₁ fragment of pre-tRNA^{Tyr}. New T₁ oligonucleotide products in *a* and *b* are labelled a-f, a, b, e and f are discussed in the text. *c* and *d* have not been analysed in detail but are likely to be derived from S₁ cleavage at the 3' terminus in oligonucleotide 18. Oligonucleotide 15 derived from the more frequent cleavage by S₁ is labelled '15 slow' and oligonucleotide 15 derived from the minor cleavage (by S₁) is labelled '15 fast'. *c*, The RNase T₁ oligonucleotides present in the larger S₁ fragment of tRNA^{Tyr}. Oligonucleotide 7 migrated along the edge of the homochromatography plate and appears as two spots. *d*, The RNase T₁ oligonucleotides present in the smaller S₁ fragment of tRNA^{Tyr}.

Pre-tRNA^{Tyr} cannot be aminoacylated *in vitro*

³²P-RNA labelled to a low specific activity was prepared and pre-tRNA^{Tyr} isolated in microgram amounts after electrophoresis in a 10% gel. The purity of pre-tRNA^{Tyr} was assessed by fingerprinting (Fig. 2).

Yeast aminoacyl-tRNA synthetases were prepared from wild-type yeast and the enzymes further purified by chromatography on DEAE Sephadex. The preparation was free of nuclease and processing activities, as degradation of precursor tRNA could not be detected after treatment with excess synthetase (data not shown).

The reaction conditions for aminoacylation of tRNA were optimised using highly purified tRNA^{Tyr} as substrate. In these conditions aminoacylation was complete. One mol of ³H-tyrosine was added per mol of mature tRNA^{Tyr}. However, using identical conditions pre-tRNA^{Tyr} could not be aminoacylated (Table 2). The inability of pre-tRNA^{Tyr} to be aminoacylated was not due to contamination; unpurified mature tRNA^{Tyr} isolated from the same gel as the precursor was a good substrate (Table 2). Mixing experiments also indicated that the pre-tRNA^{Tyr} preparation was not an inhibitor of the reaction (Table 2).

Table 2 Inability of pre-tRNA^{Tyr} to be aminoacylated

	³ H-Tyrosine c.p.m. incorporated§	
	Expt 1	Expt 2
No RNA	2,300	1,714
tRNA ^{Tyr*}	58,000	77,203
tRNA ^{Tyr†}	11,299	10,349
pre-tRNA ^{Tyr}	1,426	971
pre-tRNA ^{Phe‡}	1,601	1,083
tRNA ^{Tyr*} + tRNA ^{Tyr†}	68,768	50,487
tRNA ^{Tyr*} + pre-tRNA ^{Tyr}	71,952	52,409
tRNA ^{Tyr*} + pre-tRNA ^{Phe‡}	64,167	—
tRNA ^{Tyr†} + pre-tRNA ^{Tyr}	8,695	8,963

Aminoacyl-tRNA synthetases were prepared from 10 g of yeast cells collected in mid-log growth and lysed by grinding with a mortar and pestle in the presence of alumina. The extract was digested for 5 min with DNase (1 µg ml⁻¹) at room temperature in 50 mM Tris-HCl, pH 8.0, 5 mM MgCl₂ and 50 mM KCl. After low speed centrifugation, the crude extract was adjusted to 1 mM dithiothreitol (DTT), and centrifuged at 48,000 r.p.m. for 90 min in a Ti50 rotor. The supernatant was brought to 50 mM Tris-HCl, pH 7.9, 50 mM ammonium sulphate, 0.5 mM EDTA, 1 mM DTT, 25% glycerol and loaded onto a DEAE-Sephadex column (1.2 cm × 10 cm) equilibrated with the same buffer without glycerol. The column flow through was collected and precipitated overnight at 0°C in 70% ammonium sulphate. The precipitate was resuspended in 2 ml, 50 mM Tris-HCl, pH 7.9, 0.5 mM EDTA, 1 mM DTT and 50% glycerol. Aminoacylation of tRNAs was carried out in 10 µl reactions containing 0.04 M Tris-HCl, pH 7.9, 0.02 M MgCl₂, 0.04 M KCl, 5 mM ATP, 10 mM ³H-tyrosine (specific activity of 42 Ci mmol⁻¹) and either 100 ng (or 200 ng) tRNA for reactions containing one (or two) species of tRNA (or pre-tRNA). Using these conditions, aminoacylation is not limited by substrate. After incubation at 37°C for 10 min, aliquots were removed, spotted onto Whatman 3MM paper and precipitated with cold 10% trichloroacetic acid (TCA). Following several washes with 5% cold TCA, the paper was dried and counted by liquid scintillation. Pre-tRNA^{Tyr}, pre-tRNA^{Phe} and unpurified tRNA^{Tyr} were purified from cells labelled at 0.1 mCi ³²P-phosphate per ml.

* Pure tRNA^{Tyr}

† Unpurified tRNA^{Tyr} isolated from same 10% acrylamide gel as pre-tRNA^{Tyr}.

‡ Pre-tRNA^{Phe} isolated from same 10% acrylamide gel as pre-tRNA^{Tyr} and tRNA^{Tyr}.

§ Counting efficiency of ³H-tyrosine on Whatman 3MM paper was determined to be 3%.

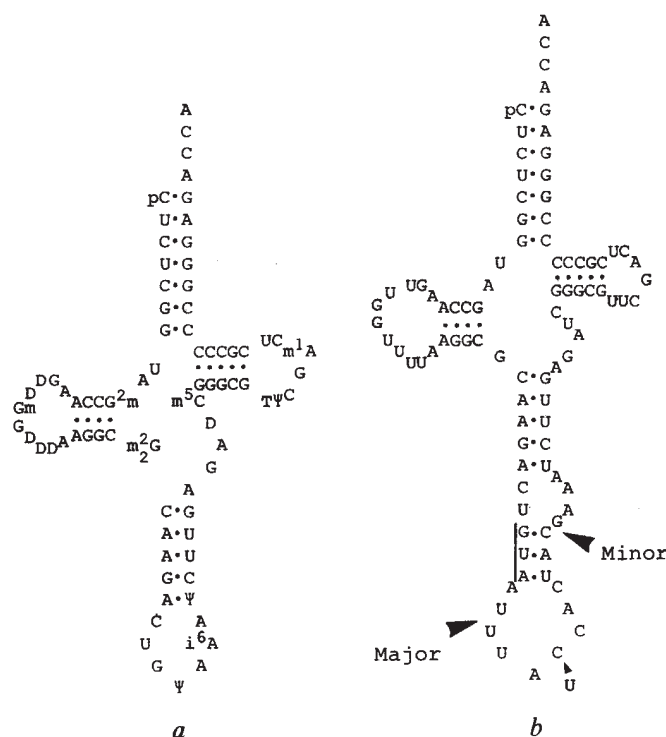


Fig. 8 Secondary structure of tRNA^{Tyr} and pre-tRNA^{Tyr}. tRNA^{Tyr} (a) is shown as previously described^{30,31}, and pre-tRNA^{Tyr} (b) is shown as predicted from the data presented. The area of the major and minor sites of S₁ cleavage are indicated by arrows. The single base change in the intervening sequence corresponding to different tRNA^{Tyr} genes is also indicated. None of the minor bases in pre-tRNA^{Tyr} are indicated. The anticodon triplet is overlined in b.

Discussion

The presence of tRNA precursors in yeast has been reported earlier²⁷. It was previously tacitly assumed that pre-tRNAs contain additional sequences at the 5' and 3' ends of the molecules as had been determined for *Escherichia coli*²⁸ and T4 bacteriophage²⁹ tRNA precursors. We have analysed in detail a particular precursor, pre-tRNA^{Tyr}, and have shown that it contains an internal sequence identical to the intervening sequence found in the gene for tRNA^{Tyr}, but no additional nucleotides at the termini. Presumably, the end of the primary gene transcript are processed rapidly during synthesis of pre-tRNA^{Tyr} to remove any additional 5' sequence and to add the 3' terminal CCA. Thus, the removal of the intervening sequence is apparently a late step in the processing pathway for tRNA at least in the mutant *ts136*. Perhaps the excision-ligase activity which removes intervening sequences is defective in *ts136* cells.

It is not known what fraction of tRNA genes contains intervening sequences. We can detect about 10 species of RNA which may be precursor tRNAs (see Fig. 1). The *in vitro* processing of all of these pre-tRNAs suggests that they all contain intervening sequences. Although some precursors may not be resolved from each other by the electrophoretic separation, it nevertheless seems that not all tRNAs can be synthesised from precursors which accumulate in the *ts136* cells used in this study. The physiological reason that some pre-tRNAs contain intervening sequences and others do not is not yet known. There may even be different routes for the synthesis of iso-accepting tRNAs, as it has been shown that tRNA^{Arg}, tRNA^{Asp} and some genes for tRNA^{Ser} do not contain intervening sequences (ref. 14 and G. Page, personal communication).

The ability to process pre-tRNAs to mature 4S RNAs *in vitro* provides a model system to study processing of pre-RNAs. We

are currently trying to purify the excision-ligase activity such that the detailed mechanism of the reaction can be studied. In the present study we have used a crude nuclei system containing contaminating cytoplasmic components and therefore cannot specify the cellular location of the excision-ligase activity. However, it seems likely that there is no major cell structural component required, as we have been able to recover the excision-ligase activity from a yeast supernatant fraction after centrifugation at 100,000g.

The excision-ligase activity resembles other enzymatic reactions which make and break nucleotide bonds. The stimulation of the reaction by ATP suggests a requirement of phosphate bond energy as in the reactions of DNA ligase, RNA ligase and DNA gyrase³⁰⁻³². The specificity of the reaction must be stringent and cannot be determined by nucleotide sequence alone (provided there is not a separate enzyme system for each sequence) as the intervening sequence is different in pre-tRNA^{Tyr} and pre-tRNA^{Phe} (refs 10, 11). Presumably, the three-dimensional structure of pre-tRNA must be such as to allow precise processing to mature tRNA.

The conformation of pre-tRNA^{Tyr} has been analysed by determining the products of cleavage by S₁ endonuclease and its structure is in general similar to that of mature tRNA^{Tyr}, as the extended anticodon loop is sensitive to S₁. However, in contrast to mature tRNA^{Tyr} the anticodon triplet of pre-tRNA^{Tyr} is not sensitive to S₁ nuclease. Previously, we had proposed that the anticodon triplet may be involved in base pairing with the intervening sequence in pre-tRNA^{Phe} (ref. 11), and the present results on pre-tRNA^{Tyr} conform to this prediction (see also ref. 14).

The lack of biological activity and the determined structural features of pre-tRNA^{Tyr} may provide a possible rationale for the existence of some specificity of sequence and conformation in tRNA intervening sequences. According to this hypothesis, the intervening sequence must contain at least a three-nucleotide stretch or codon sequence which can base pair with the anticodon triplet. Furthermore, the nucleotides flanking the codon must allow the formation of a loop structure which permits codon-anticodon interaction and provides a tertiary structure such that pre-tRNA is a specific substrate for the excision-ligase activity.

The function of the intervening sequence in the pathway for the production of biologically active tRNA is not fully understood. However two features must be accounted for in any hypothesis; pre-tRNA^{Tyr} cannot be aminoacylated until processed to tRNA^{Tyr}, and the anticodon is not accessible for hydrogen bonding. A possible function of the intervening sequence is that other processing and maturation of tRNA may be completed before removal of the intervening sequence and the assumption of biological activity. Of course, there remains the possibility that pre-tRNAs containing intervening sequences have other specific biological functions in the regulation of cell metabolism.

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A specific glycoprotein as the target site of adhesion blocking Fab in aggregating *Dictyostelium* cells

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During the acquisition of aggregation competence a new antigen appears on the surface of Dictyostelium cells. Univalent antibody fragments (Fab) against this antigen render the cells unable to form the specific type of cell adhesion which is characteristic of aggregating cells. This membrane constituent has been purified and identified as a concanavalin A-binding glycoprotein present at about 2×10^5 copies per cell.

THE molecular basis of cell-to-cell adhesion is being investigated in a variety of systems including sponge cells¹, embryos of sea urchins and mammals³, sexually differentiated cells of algae⁴ and yeasts⁵, and in fibroblasts^{6,7}. Since the studies of Moscona⁸ much work has been concentrated on the re-aggregation of trypsin-dissociated cells of the chicken neuroretina^{9–11}. Investigation of the molecular basis of cell recognition and adhesion in these different organisms and cell types may reveal some general principles underlying cell interactions¹². Compared to animal tissues *Dictyostelium discoideum* has the advantage for aggregation studies that single cells are accessible without trypsin treatment. *D. discoideum* can be grown in fermenters, where the cells acquire the ability to aggregate within several hours after the end of nutrient supply. Large quantities of homogeneous, aggregating cells are thus available.

Two types of cell assembly during aggregation

During aggregation the cells of *D. discoideum* elongate and become strongly adhesive at their ends, but they also assemble side-by-side. Both end-to-end and side-by-side adhesion can be completely blocked by univalent antibody fragments (Fab) directed against membrane antigens of aggregation competent cells¹³. The two types of cell assembly are blocked by Fab

molecules of different specificities¹⁴. The corresponding target antigens have been referred to as contact sites A and B (Fig. 1). End-to-end adhesion can also be uncoupled from side-by-side adhesion by EDTA which selectively inhibits the latter¹⁵.

Fab molecules of specificities other than those directed against 'contact sites' do not impair cell adhesion, even when they are bound at up to 2×10^6 molecules per cell to all parts of the cell surface¹⁶. Of particular importance is the finding that non-blocking Fab remains present at the actual areas of contact between the ends of adjacent cells, as shown by fluorescent-labelled Fab¹⁶. This observation suggests that Fab molecules of $35 \times 35 \times 60$ Å size can fit within the space between contiguous surfaces without impairing cell adhesion.

Purified glycoprotein reverses adhesion-blocking effect of Fab

Contact sites A (cs-A) are defined as the developmentally regulated target sites of Fab molecules that block the end-to-end attachment of aggregating cells. Cs-A can be specifically assayed by absorption of adhesion blocking Fab. The Fab is titrated with living, aggregation competent cells in the presence of 10 mM EDTA which suppresses cs-B activity almost completely. Absorption of Fab with cs-A containing fractions decreases the adhesion blocking activity, and this decrease can be quantitated by retitration of the Fab with living cells¹⁴. This immunoassay does not depend on the availability of mono-specific antisera, since it measures blockage of a specific function of the cells by certain Fab species, rather than the total number of Fab molecules bound to the cell surface. Neither does the assay depend on the preservation of contact sites in a biologically active state since only their antigenic specificity is crucial. On these grounds the assay proved to be suitable for the purification of cs-A from cell membranes of *D. discoideum*¹⁷ and of a comparable antigen in neuroretina cells¹⁸.

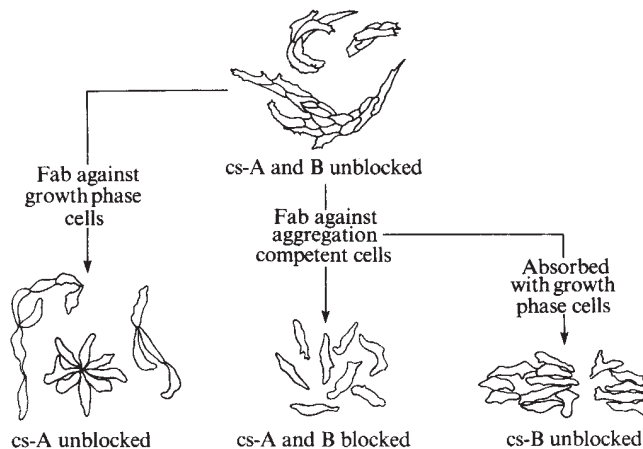


Fig. 1 Immunological analysis of the cell adhesion system in *D. discoideum*. Cells acquire the ability to aggregate within a period of several hours after the end of the growth phase. During this period specific target antigens (contact sites A) of adhesion blocking univalent antibody fragments (Fab) are expressed on the cell surface. Other target antigens (contact sites B) of adhesion blocking Fab are already present in the growth phase stage. The evidence for the diversity and developmental regulation of the contact site system is as follows. During aggregation streams of elongated cells are formed by end-to-end and side-by-side assembly (top). Fab against membrane antigens of aggregation competent cells blocks both types of contact (middle). After absorption with membranes from growth phase cells the Fab preparation still blocks the tight end-to-end contacts which are typical of aggregation competent cells (right). This demonstrates that the corresponding antigens, contact sites A, are almost absent from cells of the growth phase stage. In contrast, Fab responsible for the blockage of irregular, mostly side-by-side assembly of the cells has been removed by antigens present in growth phase cells. These antigens are the contact sites B. In agreement with these results, Fab specific for membrane antigens of growth phase cells blocks the side-by-side adhesion of aggregating cells, leaving the end-to-end contacts unaffected (left). (Data from Beug *et al.*¹⁴.)

A plasma membrane fraction was prepared from aggregation competent *D. discoideum* cells by lysis with digitonin¹⁹. The resulting ghosts were purified in a two-phase polymer system according to Brunette and Till²⁰. A glycoprotein-containing fraction was obtained by *n*-butanol extraction¹⁷. The material was further purified by DEAE-cellulose chromatography in the presence of Triton X-100 and by centrifugation on a sucrose gradient.

Purification yielded a fraction that reversed the cell dissociation produced by Fab. Dissociation was complete in about 40 min (Fig. 2). Excess cs-A added after this time enabled the cells to reaggregate within another 80 min. In the absence of Fab, purified cs-A did not exert a detectable effect on cell adhesion. Thus added cs-A apparently act by neutralisation of Fab that dissociates from its binding sites at the cell surface. Cell aggregation was thus restored, although the aggregates remained smaller than in controls (Fig. 2, bottom). Presumably the latter was due to high-affinity Fab species which did not fully dissociate from their target antigens during the experiment.

During purification a single protein co-fractionated with cs-A activity¹⁷. This was purified on a large scale. After SDS-polyacrylamide gel electrophoresis the purified protein formed a band with an apparent molecular weight 80,000 (Fig. 3).

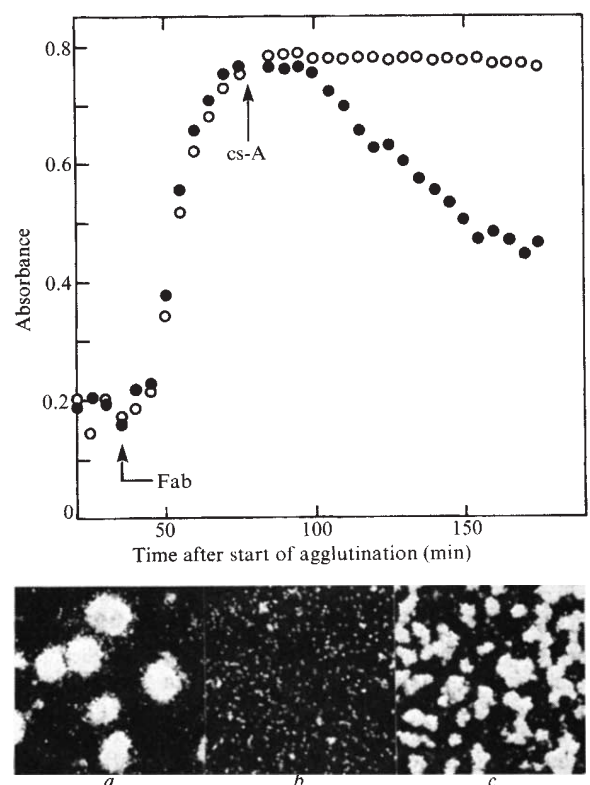
Cs-A bind to concanavalin A-Sepharose and are eluted by methyl- α -mannoside²¹. Accordingly, the 80,000 MW band of the purified material binds concanavalin A as demonstrated by staining with peroxidase as described by Parish²². For a glycoprotein the molecular weight obtained by SDS-polyacrylamide gel electrophoresis might be an overestimate. Studies with

native, deoxycholate solubilised cs-A yielded an apparent MW of between 120,000 and 130,000 on Sephadex G-200¹⁷. Loading with deoxycholate increases the apparent MW, so that it remains to be settled whether the native, detergent-treated cs-A are monomeric or dimeric.

Are contact sites A related to the carbohydrate binding protein, discoidin?

As judged by SDS-polyacrylamide gel electrophoresis, the purified cs-A are free of discoidin, a lectin thought to be implicated in cell adhesion²³⁻²⁵. The fact that the adhesion-blocking Fab is not directed against discoidin might indicate that this carbohydrate binding protein does not mediate adhesion between aggregating cells of *D. discoideum*. Alternatively, discoidin could have a function in adhesion which is not affected by Fab binding. In the latter case adhesion-blocking Fab might be directed against discoidin receptors on the cell surface²⁶ rather than against discoidin

Fig. 2 Dissociation of aggregated cells by Fab, and recovery of cell adhesiveness after addition of purified contact sites A. Aggregation-competent cells of strain v-12/M2 were washed and allowed to agglutinate at a density of 10^7 cells per ml in 17 mM Sørensen phosphate buffer pH 6.0 in conditions of standardised shear³⁵. 10 mM EDTA was added for suppression of cs-B activity. Top: dissociation and agglutination was followed in cell suspensions by recording optical absorbance as described by Beug *et al.*¹⁴. Rise of absorbance indicates increase of light scattering due to cell dissociation. At the time indicated 0.8 mg ml⁻¹ Fab directed against membranes of aggregation competent cells were added. After dissociation one of the samples (●) was supplied with purified cs-A at a final concentration of 3.5 μ g protein per ml. The cells started to agglutinate again, whereas the control cells (○) remained dissociated. Bottom: dark field photographs from the same experiment showing agglutinated cells before Fab addition (a), dissociated cells 40 min later (b), and re-agglutinated cells at 100 min after cs-A addition (c).



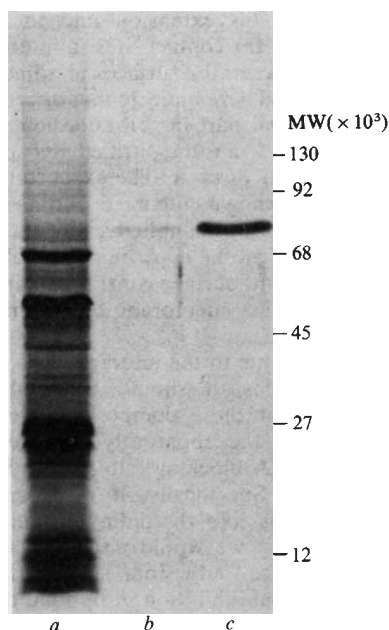


Fig. 3 SDS-polyacrylamide gel electrophoresis of a plasma membrane fraction (a) and of purified contact sites A (b, c). Samples were heated for 3 min with β -mercaptoethanol and sodium dodecyl sulphate according to Laemmli³⁶, and applied to a gradient gel of 5–15% acrylamide. The gels were stained with Coomassie blue. Total protein dispensed on the gels was 43 μ g in (a), 0.6 μ g in (b) and 6 μ g in (c). The molecular weight markers were β -galactosidase (130,000), phosphorylase (92,000), bovine serum albumin (68,000), ovalbumin (45,000), concanavalin A (27,000), and cytochrome c (12,000).

itself. Attempts to verify a discoidin-binding activity of cs-A, however, have not been successful: solubilised and partially purified cs-A did not block the carbohydrate binding site of discoidin¹⁷.

The possible function of cell surface lectins in cell adhesion has been more carefully studied in a related species, *Polysphondylium pallidum*, where Fab against the carbohydrate-binding protein, pallidin, has been reported to block cell adhesion^{27,28}. When the conditions were optimal for cell aggregation the blocking effect was very weak, even at high Fab concentrations²⁹. The same Fab completely inhibited the pallidin-induced agglutination of erythrocytes. This shows that the Fab did block the carbohydrate binding site of pallidin.

These results from our laboratory are in accord with those of Rosen *et al.*²⁸ who found that in optimally aggregating cells anti-pallidin Fab failed to produce appreciable inhibition of cell adhesion. A significant effect of anti-pallidin Fab was, however, observed under conditions called 'permissive'—after treatment of the cells with 2.5 mM 2,4-dinitrophenol²⁸ or 0.4 M glucose²⁷. For the interpretation of the data it is important to know whether the clumping of cells treated in this way is related to cell aggregation as it normally occurs during development.

There is no question that in normal conditions, which are 'nonpermissive' for cell dissociation by anti-pallidin Fab, adhesion of *P. pallidum* cells can be completely inhibited by Fab directed against membrane antigens other than pallidin. These are the antigens called contact sites. In summary, blockage of cell adhesion by Fab has provided no evidence for the importance of pallidin in those types of cell adhesion which are related to morphogenesis. In *P. pallidum*, as well as in *D. discoideum*, the principal target antigens of the adhesion-blocking Fab species are definitely different from the carbohydrate-binding proteins pallidin and discoidin.

Immunospecificity of the purified contact sites

All Fab species that block cs-A activity are directed against the single glycoprotein fraction shown in Fig. 3c. To establish this, a highly polyspecific Fab preparation was quantitatively absorbed with the purified glycoprotein. The Fab was prepared from the pooled hyperimmune sera of 22 rabbits¹⁶. This is important because individual rabbits often select from an antigen mixture certain antigens to which they form antibodies. The finding that only one antigen absorbs the adhesion blocking Fab could mean then that antibodies had been formed only against this antigen. Pooling of antisera makes the Fab preparation polyspecific enough to render this possibility unlikely.

The Fab was first absorbed with the fraction shown in Fig. 3c, and then tested with aggregation competent cells in the presence of EDTA for the remaining adhesion-blocking activity. Absorption with sufficiently high quantities of cs-A completely removed the adhesion-blocking activity, indicating that all immunodeterminants to which cs-A-blocking Fab will bind were present in the purified material (Figs 4 and 5).

Cs-A are species-specific in the sense that anti-cs-A Fab does not block cell adhesion in *P. pallidum*, and conversely Fab against contact sites of *P. pallidum* does not block the EDTA-stable type of cell adhesion in *D. discoideum*. Mixed cells of the

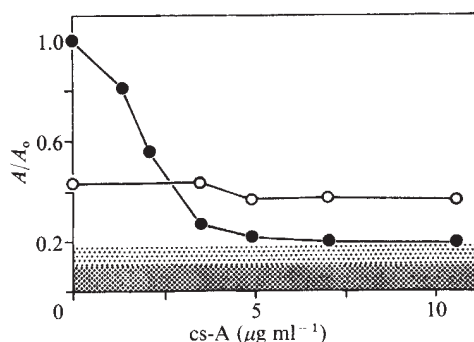


Fig. 4 Absorption of Fab directed against cs-A (●) or cs-B (○) by purified cs-A. Ordinate: optical absorbance was taken as a measure of cell dissociation (see legend to Fig. 2). The values were normalised to optical absorbance in completely dissociated but otherwise identical samples, such that a value of $A/A_0 = 1$ indicates complete cell dissociation in a test sample. Abscissa: quantity of purified cs-A used for Fab absorption. Constant amounts of Fab were mixed with cs-A, and the adhesion blocking activity of the Fab was retitrated using living cells. Absorption of anti-cs-A Fab (●): aggregation competent cells of strain v-12/M2 in barbital buffer pH 7.3 were used as test cells. The Fab was directed against membranes of aggregation competent cells. The assay was made absolutely specific for cs-A by the addition of 10 mM EDTA and by exhaustive preabsorption of the Fab with growth phase cells. Fab was used at 0.8 mg ml⁻¹ which in the absence of cs-A completely dissociated the cells. Conditions as in ref. 14. The upper border of the dotted area indicates optical absorbance in controls to which neither Fab fragments nor cs-A were added. In the presence of Fab plus high concentrations of purified cs-A the cells agglutinated to the same extent as in controls, indicating complete absorption of anti-cs-A Fab by the purified material. Absorption of anti-cs-B Fab (○): exponential growth phase cells of strain v-12/M2 were washed and immediately used in the absence of EDTA for the assay of cs-B activity. The Fab was directed against the membranes of aggregation competent cells. Because of the absence of cs-A from the surface of growth phase cells the assay was specific for cs-B. The quantity of Fab used, 1.6 mg ml⁻¹, inhibited cs-B activity in the test cells to a degree where changes of the adhesion blocking activity of the Fab were most sensitively detected. Cs-B specificity was practically absent from the purified cs-A fraction as evidenced by the negligible reduction of the adhesion blocking activity by high concentrations of cs-A. The optical absorbance of aggregated control cells to which no Fab or cs-A were added is indicated by the upper border of the densely dotted area.

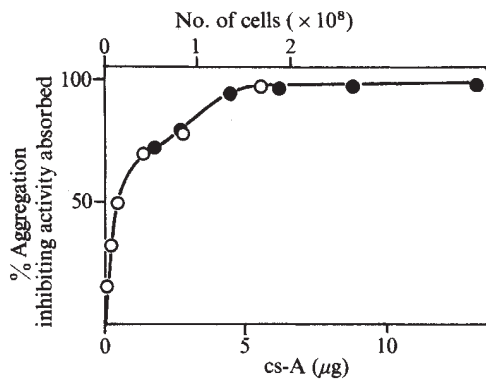


Fig. 5 Quantification of contact sites A on the cell surface by comparison of the Fab absorbing activity of intact cells (○) and of the purified cs-A fraction (●). Ordinate: in assays like those shown in Fig. 4 the absorption of anti-cs-A Fab was determined and the % adhesion blocking activity absorbed was calculated according to Fig. 3 of ref. 14. The Fab was directed against membranes of aggregation competent cells and was pre-absorbed with growth phase cells. Upper abscissa: absorption of Fab by living, aggregation competent cells (○); these data are from Fig. 4 of ref. 16. Lower abscissa: absorption of Fab from the same batch of antisera by purified cs-A (●). Quantities are in μg protein in the cs-A fraction determined according to Wang and Smith³⁷ with bovine serum albumin as standard. Quantities of cells and cs-A are calculated per mg Fab.

two species sort out within the aggregates into separate clusters²⁹. Thus species specificity of contact sites might have behavioural consequences.

The purified cs-A showed no significant cross-reaction with Fab against cs-B (Fig. 4). This result suggests that the two types of cell adhesion observed in aggregating *D. discoideum* cells (Fig. 1) are blocked by Fab molecules directed against different cell surface molecules.

Comparison of Fig. 3a and c indicates that cs-A do not belong to the predominant proteins of the plasma membrane. As quantitated by Fab absorption, the protein content of cs-A amounts to about 1% of the total membrane protein. Also as a concanavalin A-binding glycoprotein the cs-A molecule is one among many others. Aggregating cells of *D. discoideum* contain at least 35 different concanavalin A-binding membrane proteins³⁰. At least 15 of them are exposed to the extracellular space, as revealed by iodine labelling of intact cells³¹.

Contact sites A are minor constituents of the cell surface

Various data provide further evidence that the cells are not covered by a continuous surface coat formed by cs-A. The availability of cs-A in a purified state and an estimate of 80,000 for their molecular weight allows us to calculate the number of cs-A monomers exposed on the surface of aggregation competent cells. In Fig. 5 the values of the abscissa have been adjusted so that Fab absorption by intact cells and by purified cs-A can be compared. The graph indicates that a quantity of 33 fg cs-A CS-A protein is present on a single cell; this corresponds to 2×10^5 cs-A monomers (if no correction is made for their carbohydrate content). This number is equivalent to only 5×10^2 cs-A monomers per μm^2 surface area for a rounded cell of 11 μm diameter. Fab against these sites covers less than 2% of the total surface area of the cells¹⁶.

Data obtained from electron microscopy with ferritin labelled antibodies also indicate that cs-A are interspersed on the cell surface between other antigens³². Discrete antigenic sites which are specific for aggregation competent cells extend perpendicularly to the membrane up to 40 Å beyond a carbo-

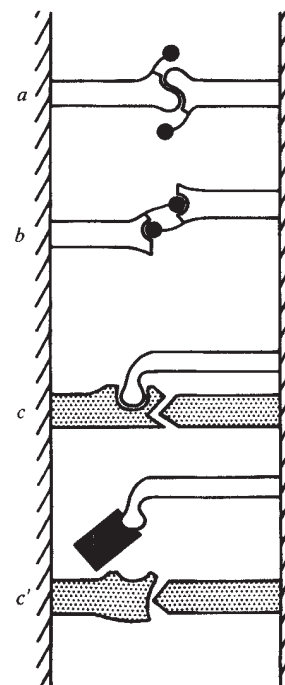
hydrate layer of fairly uniform thickness which is formed from glycosphingolipids^{33,34}. This extension into the intercellular space might be sufficient for contact sites to interact with each other in the midline between the surfaces of adjacent cells.

The results summarised here indicate that discrete loci on the cell surface play a critical part in cell adhesion, and that the material present at these loci is the purified glycoprotein carrying the immunospecificity of cs-A. The exact function of cs-A in cell adhesion is not known. Figure 6 outlines three possibilities, chosen for reasons of simplicity. Since a single glycoprotein apparently binds all the cs-A blocking Fab species, the minimal requirement is one surface constituent which mediates cell-to-cell linkage by trans-membrane homodimer formation (Fig. 6a, b).

If cell adhesion were due to the interaction of two different, complementary molecules, it should be inhibited by the inactivation of either of these components. It is therefore difficult to explain why the apparently homogeneous glycoprotein binds all the cs-A blocking Fab species from a highly polyspecific antiserum. Specifically, if receptors for carbohydrate-binding proteins like discoidin or pallidin were to function as contact sites, we would also expect Fab against these lectins to inhibit cell adhesion. However, anti-pallidin Fab exerted only minimal inhibition in optimal conditions for cell aggregation, although the Fab blocked the interaction of the lectin with receptors on the surface of erythrocytes²⁹.

More complicated models like the three-component system shown in Fig. 6c and c' make allowance for the possibility that cell-to-cell binding is mediated by ligands which interact with high affinity: possibly too high for Fab against their recognition

Fig. 6 Some possibilities how contact sites A might function in cell adhesion. In (a) and (b) the membranes of adjacent cells are held together by the contact sites, in (c) and (c') contact sites are thought to have a regulatory role. Contact sites are defined as the target sites of adhesion-blocking Fab. They are drawn as open symbols with their carbohydrate moieties as closed circles. Cell surface structures regulated by contact sites are dotted, adhesion blocking Fab is shaded. a, Trans-membrane dimer formation by protein-protein association. b, Dimer formation by mutual protein-carbohydrate interaction. c, Cell adhesion, here symbolised as the interaction of two complementary cell surface constituents, might require activation by a third component. c', Blocking of this component by Fab would bring the cells in a non-adhesive state.



sites to be able to interfere. If this were the case, the adhesion-blocking Fab might be directed against a regulatory site. Availability of the contact site material in a purified state should contribute to our understanding of its biological function.

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letters to nature

Should Hubble's constant be doubled?

LYNDEN-BELL¹ has argued that Hubble's constant H_0 should be doubled, from its present notional value of $55 \text{ km s}^{-1} \text{ Mpc}^{-1}$ to $110 \text{ km s}^{-1} \text{ Mpc}^{-1}$. He claims that with this value of H_0 the apparent superluminal expansions in compact radio sources can be explained in a more consistent manner than previously, by means of his model of the expansion based on the old 'light echo' theory used to explain the Nova Persei phenomenon of 1901. Lynden-Bell's conclusion is heavily dependent on his special interpretation of the expansion phenomena in the source 3C120. Here I revert to the interpretation previously presented by Cohen *et al.*² and use a more general 'energy echo' model that allows the primary energy ejected from the source nucleus to be propagated with a speed $v \leq c$, with Lynden-Bell's case $v = c$ as a limiting possibility. The primary energy may therefore be in the form of relativistic particles, plasmoids, shock waves or photons.

Table 1 Data for superluminal radio sources

	z	$\dot{\chi}$ (arc ms yr ⁻¹)
3C273	0.158	0.32
3C120 (i)	0.033	1.80
3C345	0.595	0.17
3C120 (ii)	0.033	2.90
3C279	0.538	0.27

Table 2 Calculations for superluminal sources for $v = c$

	(a) $H_0 = 55 \text{ km s}^{-1} \text{ Mpc}^{-1}$		(b) $H_0 = 110 \text{ km s}^{-1} \text{ Mpc}^{-1}$	
	$q_0 = 0.05$		$q_0 = 0.05$	
	v_s/c	θ_c	v_s/c	θ_c
3C273	4.049	29.60°	2.024	81.17°
3C120 (i)	5.042	23.37°	2.521	52.50°
3C345	6.976	16.66°	3.488	34.99°
3C120 (ii)	8.123	14.25°	4.062	29.50°
3C279	10.180	11.33°	5.090	23.14°

Let the axis of ejection make angle θ with the line of sight and the velocity of ejection be v . The primary energy moving outwards in opposite directions through the surrounding stationary plasma will generate two secondary source components. These will propagate outwards along the axis with speed v in accordance with the echo concept. Then it may be shown that their apparent speed of separation transverse to the line of sight is

$$v_s = \frac{2v \sin \theta}{1 - (v^2/c^2) \cos^2 \theta} \quad (1)$$

This reduces to Lynden-Bell's formula $v_s = (2c)/\sin \theta$ if $v = c$.

The data for a source provide an 'observed' value of v_s regardless of the model in the manner described by Lynden-Bell¹. Thus in the usual notation ($\dot{\chi}$ is the observed rate of angular separation of components):

$$v_s = (1+z)\dot{\chi} \quad \left. \begin{array}{l} \text{where} \\ \delta = \frac{c}{H_0 q_0^2} \cdot \frac{[q_0 z + (q_0 - 1)\{(1 + 2q_0 z)^{1/2} - 1\}]}{(1+z)^2} \end{array} \right\} \quad (2)$$

If an observed value of v_s is substituted into (1) then it may be shown that if $v_s \geq 2c$ (superluminal case) there is a circular cone bounded by the generators $\theta = \theta_c = \sin^{-1}(2c/v_s)$ inside and on which any direction θ and corresponding $v \leq c$ will provide a solution to (1). In Lynden-Bell's case the direction θ_c is the only possible orientation.

Table 1 presents the observed primary data for the five cases discussed by Cohen *et al.*² 3C120 (i) and 3C120 (ii) distinguish the two successive eruptions as interpreted by these authors. Table 2 shows for each source the value of v_s obtained from equation (2): (a) for $H_0 = 55 \text{ km s}^{-1} \text{ Mpc}^{-1}$ and (b) $H_0 = 110 \text{ km s}^{-1} \text{ Mpc}^{-1}$. Also shown is the value of $\theta = \theta_c$ calculated from (1) when v has the limiting value c .

It will be seen that in case (a) the directions of the ejection tubes all lie within a cone of semi-angle 30°. On the other hand the mean expected value of θ for a complete source sample would be 60°; and so in case (a) all the sources seem highly

Table 3 Predicted distributions of $v_s(\theta)$ for various propagation speeds v , and maximum values

θ	2	5	10	15	20	30	40	60	75	90	v_s/c (max)	θ (max)
v/c												
0.950	0.67	1.59	2.65	3.11	3.20	2.94	2.60	2.12	1.95	1.90	3.20	19.19°
0.996	7.56	11.2	9.13	6.93	5.49	3.89	3.06	2.29	2.06	1.99	11.19	5.15°
1.000	57.3	22.9	11.5	7.73	5.85	4.00	3.11	2.31	2.07	2.00	∞	0°

Fraction of sources $\leftarrow \frac{1}{16} \rightarrow \leftarrow \frac{1}{16} \rightarrow \leftarrow \frac{1}{8} \rightarrow \leftarrow \frac{1}{4} \rightarrow \leftarrow \frac{1}{2} \rightarrow$

exceptional. In case (b) the last three appear exceptional. The probability of a tube being in the range $0 < \theta < 35^\circ$ is 0.18 (less than a 1 in 5 chance), yet three cases fall into this category in (b). Even smaller values of θ would be required if $v < c$. There is therefore no compelling case for changing H_0 on the basis of the data of Table 1.

If $v = c$ we cannot explain 3C120 as two successive eruptions as interpreted by Cohen *et al.*² in Table 1, because Table 2 shows that two different values of θ are required, so the tube would not be fixed in the nucleus. However, if we allow $v \leq c$ we can explain 3C120 (i) and (ii) by choosing two different values of v for the same value of θ . From Table 2, if $H_0 = 55 \text{ km s}^{-1} \text{ Mpc}^{-1}$ we must choose $\theta \leq 14.25^\circ$. For example, the choice $\theta = 13^\circ$, $v_1 = 0.980c$, $v_2 = 0.998c$ will explain the phenomena in accordance with equation (1), and conform to the considerable evidence and opinion that θ should be fixed³⁻⁶. If $H_0 = 110 \text{ km s}^{-1} \text{ Mpc}^{-1}$ we require $\theta \leq 29.50^\circ$. Taking $\theta = 27^\circ$ we require $v_1 = 0.918c$, $v_2 = 0.990c$.

It is useful to look at equation (1) from the inverse point of view. If we specify a value of $v \leq c$ then (1) gives the value of v_s (not necessarily superluminal) that would be observed for a given θ in the range $0 < \theta \leq \pi/2$. To have at least one direction in which $v_s > 2c$ requires $v > 0.866c$. For $v < c$, v_s starts at zero at $\theta = 0$, rises to a strict maximum in $0 < \theta < \pi/2$ and then decreases to the value $2v$ at $\theta = \pi/2$.

Table 3 shows the predicted values of v_s at various θ for the cases $v/c = 0.950$, 0.996 and Lynden-Bell's case $v/c = 1$. It shows also the maximum value of v_s and the corresponding value of θ . Note that these results are independent of what value H_0 takes.

In all three cases of v the values v_s around the mean value of θ , namely 60° , are just over $2c$ with Lynden-Bell's case the highest. As v rises the strict maximum of v_s ($v < c$) rises steeply and takes place at values of θ of relatively low probability in a complete sample. At $v = 0.996c$ superluminal character is evinced over practically the whole range of θ . To explain exceptional cases like 3C345, 3C120 (ii) and 3C279 ($v_s \geq 3.5c$ even if H_0 is doubled) requires a value for v not less than 96% of the velocity of light. Since we might characterise the $v_s(\theta)$ distribution for a given $v < c$ by the ratio $v_s(\theta)/v_s(\text{max})$ it is in principle possible to make a qualitative test of complete sample data even if a wrong value is taken for H_0 . When the relevant v , or range of relevant v , had been determined in this way H_0 could be ascertained by fitting the data to the correct absolute distribution of $v_s(\theta)$ for the v so determined. For example, if the observations yielded relative distributions which indicated that $v \leq 0.996c$ then H_0 would have to fit the result $v_s(\text{max}) \leq 11.19c$ (Table 3).

It has been estimated³ that 10% of all double radio sources have compact radio components associated with the nucleus of the parent body. Recent tests of large complete samples of radio sources have shown that up to 35% appear to have compact components⁷. The sources of Table 1, which have received detailed observation by very long baseline interferometry (VLBI), seem to be extreme cases not likely to be typical of a complete sample, according to the analysis presented here. A possible explanation is that for superluminal character to be observed at all by VLBI in its present early

development probably requires very outstanding cases to clearly demonstrate the phenomenon. Future VLBI observations over a wider field should provide the necessary information to isolate the relevant kinematics and the underlying physics, as well as providing a measure of Hubble's constant.

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Low mass portion of the galaxy clustering spectrum

To understand the physical processes which controlled the formation of individual galaxies and clusters of galaxies, it is very important to determine the relative numbers of clusters over a wide range of masses. Although this present day clustering spectrum may not perfectly match the primordial spectrum, one hopes that the major features of the latter might be deduced from the observations. For the massive end of the clustering spectrum, the catalogue of Abell¹ can be analysed to provide the necessary data². For the faint end there are many problems, both observational and theoretical. The observational problems arise if attempts are made to specify group or cluster membership when insufficient data are available. These problems can be avoided by using a complete redshift survey. We have previously presented a large redshift survey of galaxies in a 260 deg^2 region of the sky in the direction of the rich clusters Coma and A1367. We found eight groups and clouds which lie in the $4.2 \times 10^4 \text{ Mpc}^3$ (assuming $H_0 = 50 \text{ km s}^{-1} \text{ Mpc}^{-1}$) between the local supercluster and the Coma/A1367 supercluster. We use this new survey data here to determine the low mass portion of the clustering spectrum.

Although the data are limited by the small number of groups studied, they have the following important features. (1) The data are uniform. Redshifts and magnitudes have been determined to a fixed apparent magnitude over the whole survey region which allows us to calculate intrinsic cluster masses, luminosities, and richnesses. (2) Because the redshift sample is very nearly complete, we need not guess at foreground and background corrections for individual groups. (3) Because of the completeness of the sample, we can be confident that we

have identified all of the groups and clouds in our survey area. (4) The galaxy systems that we include in the present study lie outside superclusters. We have shown³ that the number of groups seen today within the Coma/A1317 supercluster probably does not accurately represent the primordial distribution as tidal forces produced by nearby, massive clusters probably disrupted the least dense systems. Furthermore, the present masses of those supercluster groups that have survived are likely to be less than their initial values as some group members may well have been stripped away in the same manner. Although supercluster systems may be of little use in determining the primordial spectrum of density irregularities, the spectrum of clusters within superclusters may lead to an understanding of how massive clouds fragment during their collapse (assuming that this collapse precedes the formation of galaxies—as suggested in refs 4–6). We suggest that the clustering spectrum of systems outside superclusters should be determined separately from the spectrum within superclusters.

For the eight groups and clouds in our survey volume, we find the average luminosity (by extrapolating down to $M_p = -15.0$ to remove the Malmquist⁷ bias, that nearby galaxies are surveyed to intrinsically fainter limits than distant galaxies) to be $2.3 \times 10^{11} L_\odot$ and that the frequency of occurrence of such systems is approximately 1 per $5.5 \times 10^3 \text{ Mpc}^3$. These quantities shown in Fig. 1 give the location of groups in the general clustering spectrum. Figure 1 also shows a line which represents the results calculated by Chincarini² for the rich clusters. The line joining our single point to the rich cluster spectrum has a different gradient from that representing rich clusters. The difference in slopes is not statistically significant using current data but indicates that the spectrum may change shape in the range $L \sim 10^{12} L_\odot$.

The final point which we add to the spectrum is of great importance. Our previous analysis³ shows that apart from

superclusters the number of truly isolated ('field') galaxies is very small. In the survey volume containing eight groups we found only four galaxies that might be considered isolated; the other 42 galaxies are clearly members of the eight groups. The four isolated galaxies contribute an average luminosity of $\sim 5 \times 10^9 L_\odot$. Because any of these four galaxies might be outlying members of the groups, we plot this final point in Fig. 1 as an arrow representing an upper limit to the single galaxy density. (We note that three of the four galaxies may have originated in the NGC4793 cloud, and Tifft and Gregory⁶ have recently found additional galaxies with redshifts similar to the fourth galaxy. Therefore, in reality there may be no 'field' objects at all.)

As no single straight line can fit all the data points, we suggest that a low mass cutoff truncates the clustering spectrum at a group luminosity of $\sim 10^{11} L_\odot$. Assuming a mass to light ratio of approximately 100, this cutoff corresponds to a mass of $10^{13} M_\odot$.

Gott and Turner⁹ have made an independent determination of the clustering spectrum. They suggest that the number density of groups is a monotonically decreasing function of total luminosity for all luminosities studied. The implication is that single galaxies are merely the faint end of the cluster luminosity function.

Clearly, for the faint end of the clustering spectrum, our results conflict with those of Gott and Turner⁹. The differences are probably because: first, The Gott and Turner data are based on relatively few observed redshifts and hence do not satisfy features (1)–(3) already listed. Second, the Gott and Turner groups are a mixture of systems located both within and outside the local supercluster and hence do not satisfy condition (4). Third, Gott and Turner claim that it makes no sense to speak of groups without defining *a priori* a density enhancement, χ (in this picture, groups must have an average density, ρ , satisfying $\rho > \chi \bar{\rho}$, where $\bar{\rho}$ is the average density of a fair sample of the Universe). However, the Gott and Turner definition is based on the hypothesis that groups are nothing more than density irregularities surrounded by 'field' which has a density near $\bar{\rho}$. As we have found³ that the average nearest neighbour separations for the eight groups considered here are $7 \times \bar{R}_h$, where $\bar{R}_h$ is the mean harmonic radius of the groups, and that the intergroup space is nearly devoid of galaxies, we conclude that the distribution of groups outside superclusters is characterised by very large density enhancements compared to that of the intergroup space (not compared to the average of both voids and groups).

Because our data are based on the first large scale sample to be studied in three dimensions, we believe that our results represent the true distribution of galaxies more accurately than do earlier studies of the two dimensional distribution. A preliminary study of the three dimensional distribution of galaxies in the direction of another supercluster¹⁰ supports the suggested paucity of single galaxies outside clusters or superclusters. Although additional redshift surveys ought to be completed—particularly in regions that do not contain rich clusters, we conclude that the turnover in the clustering spectrum is real.

We interpret the break in the clustering spectrum at $L \sim 10^{11} L_\odot$ ($M \sim 10^{13} M_\odot$) as implying that individual galaxies (and doubles, triples and so on) were formed by a different mechanism from that which formed groups and clusters. The rare instances of isolated, single galaxies do not seem to be the degenerate case of clusters, but instead these galaxies were probably expelled from clusters or groups. Bright galaxies were probably formed only in those regions of space where at least 10 or 20 other bright galaxies were also formed.

Figure 1 shows a possible shape for the primordial cutoff which occurs between the luminosities of groups and single galaxies. This observationally based result may be closely related to the repeated suggestions^{11–14} made from a theoretical viewpoint that below the mass limit $\sim 10^{13} M_\odot$, primordial density irregularities are damped out before the epoch of

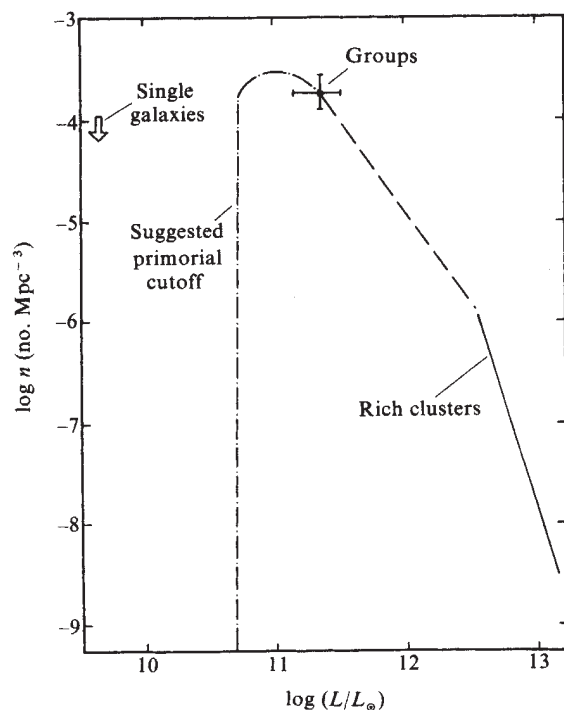


Fig. 1 The spectrum of galaxy clustering. The line representing rich clusters is taken from the data of Chincarini². We add a point representing groups and an upper limit to the number of single galaxies seen in our sample. No straight line can represent all of these data. We suggest that there was a sharp cutoff in the primordial spectrum, that is, no single galaxies were created outside clusters or groups. A possible shape for this cutoff has been sketched in. The dashed line joins our single point to the rich cluster spectrum.

recombination (this applies to both adiabatic irregularities and to photon turbulence irregularities). Doroshkevich *et al.*⁴ have presented from theoretical considerations an approximate form for the adiabatic spectrum of density irregularities, and their results are quite similar in form to the observations shown in Fig. 1. Liang¹⁵ has completed a more detailed analysis of this problem, and concludes that not only should the spectrum show a sharp falloff for masses $<10^{13} M_{\odot}$ but that somewhere above a certain mass limit the slope of the spectrum should steepen. Although the break in our spectrum also supports the latter suggestion, such details cannot be confirmed using the preliminary data presented here. Hopefully, two to three additional redshift surveys will provide sufficient data for defining the clustering spectrum properly.

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Galaxy clustering spectrum and cosmic luminosity

A PRELIMINARY correlation between richness class¹ and total cluster luminosity can be obtained from the sample studied by Oemler² (see also ref. 3), the two quantities being related by the equation:

$$\log(\text{richness}) = 1.21 + 1.13 \log \langle L/L_{\odot} \times 10^{-12} \rangle$$

where richness is given by the mean of the counts as defined for each richness class by Abell. We show here that the correlation is not a very strong one due to the dispersion intrinsic to each richness class and, above all, to the fact that there are no clusters of richness class 4 in Oemler's sample and only one cluster of richness class 5 has been found in the volume searched by Abell, about 10^9 Mpc^3 .

The number of clusters for each richness class in the complete sample correlates with the total luminosity in a manner which is well approximated by (Fig. 1)

$$\log N = 5.69 - 4.55 \log \langle L/L_{\odot} \times 10^{-12} \rangle$$

(standard error of estimate of $\log N$ on $\log L/L_{\odot}$ is 0.2; standard error of the regression constant is 0.49 and standard error of the regression coefficient is 0.54).

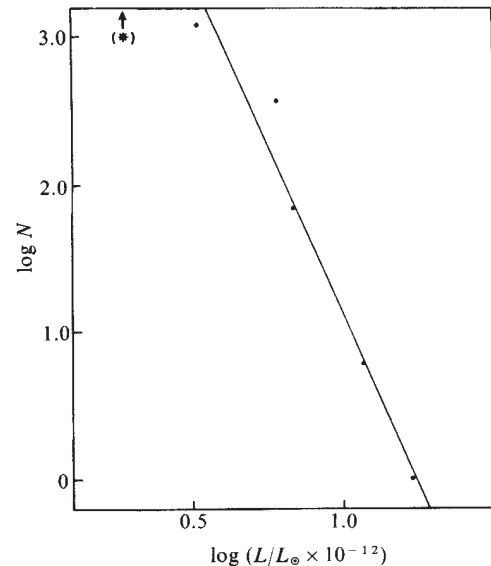


Fig. 1 Numbers of clusters per Abell's richness class as a function of the cluster mean total luminosity (see text).

Using the catalogue of Turner and Gott⁴ (TG), one observes a much lower number of groups than expected from the above equation ($L_{\text{group}} \approx 0.245 \times 10^{12} L_{\odot}$). The cluster luminosity function, therefore, may reach a plateau and decrease again toward the low luminosity end. Gregory and Thompson⁵ give another estimate for a low luminosity point of the cluster-group luminosity function.

The total cosmic luminosity due to each richness class is shown in Fig. 2, the total luminosity of class 4 was estimated by interpolation. As can be seen, the least luminous clusters make the largest contribution due to their larger number. An obvious question is then whether even less rich clusters or groups might contribute even more to the total cosmic luminosity.

Clusters of richness class 0 are not in the complete sample given by Abell. In his catalogue the number is less than (and probably $\sim 85\%$ of) the richness class 1. This would give a small contribution to the cosmic luminosity as estimated in Fig. 2 (square point). However, Abell (personal communication) points out that, due to the incompleteness of the sample, the ratio class 0/class 1 could be of the order of 3 to 6, and he would not rule out even 10 or more.

To estimate the contribution of groups of galaxies we depend on the assumed model for their distribution and on surveys

Table 1 Estimate of various contributors to the cosmic luminous density

Object	$L/L_{\odot}$ (10^{12})	Total luminosity $L_{\odot} (\text{Mpc}^{-3})$	Notes
Cluster			
Richness 5	17.0	1.78×10^4	
Richness 4	(11.75)	7.24×10^4	
Richness 3	6.85	4.79×10^5	
Richness 2	5.96	2.40×10^6	
Richness 1	3.28	4.17×10^6	
Richness 0	2.45	2.5×10^6	Uncertain
Groups outside supercluster	~ 0.2	3×10^6	Uncertain ($\sim 0.3 \times 10^6$ 'isolated?')
Groups and galaxies in supercluster	~ 0.2	$\sim 3.3 \times 10^7$	Very uncertain (2×10^6 Coma outskirts)
Single galaxies			Very low

which are essentially limited to the local supercluster⁴⁻⁷. Superclusters seem to be the major constituent of the Universe^{8,10,15} as suggested by the observed segregation of redshifts⁸⁻¹³ and by the low density, or absence of field galaxies¹⁴. It is reasonable to assume that the luminosity due to groups in superclusters is similar to the luminosity due to groups in the local supercluster. A preliminary estimate of the number of superclusters can be obtained from the data of Rood¹⁵. The ratio of superclusters to rich clusters for distance class 0-2 is somewhere between 19 and 78%; for distance class 3 the ratio is between 18 and 67%. As a conservative estimate we can then assume that the number of superclusters is about 50% of the number of rich clusters (see also ref. 15).

Using Holmberg's luminosity function for groups ($H = 50 \text{ km s}^{-1} \text{ Mpc}^{-1}$), which refers to a not very well defined volume of space, we obtain:

$$L (\text{local supercluster's groups}) \approx 9.71 \times 10^8 L_{\odot} \text{ Mpc}^{-3}$$

and using the survey of de Vaucouleurs⁷

$$L (\text{local supercluster's groups}) \approx 7 \times 10^8 L_{\odot} \text{ Mpc}^{-3}$$

The best defined sample is, in some aspects, the one published by TG^{4,17}. The surveyed volume is about $1.84/4\pi = 0.14$ of the total volume and the group distribution is shown in Fig. 3. Including only groups at distances $d \leq 60 \text{ Mpc}$, the luminous density is

$$L (\text{local supercluster's groups}) \approx 3.3 \times 10^8 L_{\odot} \text{ Mpc}^{-3}$$

Accepting a conservative value of $L (\text{LSG}) \sim 5 \times 10^8 L_{\odot} \text{ Mpc}^{-3}$ we obtain a cosmic luminous density due to supercluster groups of: $L \approx 5 \times 10^7 L_{\odot} \text{ Mpc}^{-3}$ (55 groups in 14% of the volume, mean luminosity per group $15.06 \times 10^{10} L/L_{\odot}$ and about 8.76×10^{-7} superclusters Mpc^{-3}). There are uncertainties due to the local enhancements in density, the outskirts of the Virgo cluster and the extremely uncertain estimate of the number of superclusters.

Twenty-three groups in the TG sample are at a distance between 60 and 140 Mpc, Fig. 3. These groups, which are outside the local supercluster, give a contribution to the luminous density of: $L_{\text{groups}} \approx 3 \times 10^6 L_{\odot} \text{ Mpc}^{-3}$.

A second estimate of the supercluster luminosity for non-cluster galaxies can be obtained from Chincarini and Rood.^{8,9} For the Coma supercluster there are about 100 galaxies ($m_{pg} \leq 15.1$) between 3 and 15° from the cluster centre, and 217 objects between 0 and 3° . Thus, we find in the corona about 50% of the number of galaxies in the cluster core. Such a separation is somewhat uncertain; for instance, using 1.67° we

Fig. 2 Cosmic luminous density of clusters as a function of cluster luminosity. Circled points indicate classes 1, 2, 3 and 5; the crossed point indicates class 4; the squared point indicates class 0.

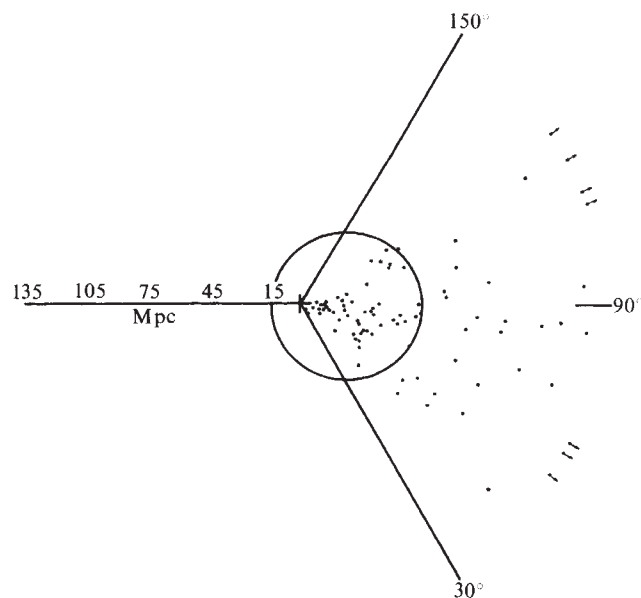
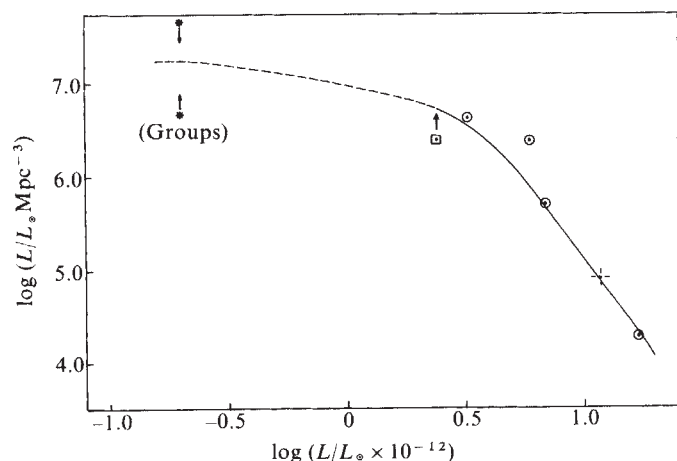


Fig. 3 Distribution of groups as a function of distance and supergalactic longitude.

would have a ratio corona/core ≈ 0.90 , therefore, we assume that such ratio is between 0.5 and 1.0. We obtain

$$L (\text{supercluster corona}) \geq 2 \cdot 10^6 L/L_{\odot} \text{ Mpc}^{-3}$$

We do not know yet whether the luminosity due to the corona of a supercluster is somehow related to the cluster richness class.

'Isolated' galaxies in the TG catalogue (although some might belong to distant clusters) contribute less than 50% of the groups. Single galaxies outside superclusters have been discussed elsewhere¹⁴ and we can disregard their contribution.

The mean value of the supercluster luminosity outside clusters would therefore be about

$$L \approx \frac{1}{2} (2 \times 10^6 + 5 \times 10^7 + 1.3 \times 10^7) = 3.3 \times 10^7 L_{\odot} \text{ Mpc}^{-3}$$

and the uncertainty of the above estimate is quite evident.

A summary of the preliminary luminous density estimates is given in Table 1. The total cosmic luminosity is then:

$$L_T = 4.6 \times 10^7 L_{\odot} \text{ Mpc}^{-3} \quad \text{All contributions in Table 1.}$$

$$L_T = 1.5 \times 10^7 L_{\odot} \text{ Mpc}^{-3} \quad \text{Assuming a Coma corona}$$

luminosity for superclusters.

$$L_T = 9.6 \times 10^6 L_{\odot} \text{ Mpc}^{-3} \quad \text{w/o groups, only clusters}$$

and probably about

$$L_T \approx 3 \times 10^7 L_{\odot} \text{ Mpc}^{-3}$$

Note that, if we use the counts in the Shapley-Ames catalogue and the equation derived from them by Holmberg¹⁸,

$$N(<m) = 1.35 \times 10^{-9} \times 10^{0.6m}$$

we derive, using Abell's galactic luminosity function (see also ref. 19).

$$L = 1.5 \times 10^8 L_{\odot} \text{ Mpc}^{-3}$$

and from TG, using magnitudes from the Zwicky catalogue,

$$L = 4.7 \times 10^7 L_{\odot} \text{ Mpc}^{-3}$$

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Resonance orbits and the rings of Uranus

SOON after the discovery of the rings of Uranus¹⁻³ it was suggested⁴ that the ring system might be determined by the perturbations of the satellite pairs Ariel-Titania and Ariel-Oberon. Aksnes⁵, and Goldreich and Nicholson⁶ have questioned this hypothesis; the latter showing that the strongest perturbations on the ring system can be expected to arise from the satellite pair Miranda-Ariel and the 4:1 resonance of Miranda. Goldreich and Nicholson also pointed out that the differences in the ring radii α - β , γ - δ and ε_1 - ε_2 are known to a high degree of accuracy (typically $\sim \pm 15$ km) which is considerably better than the absolute ring radii. My calculations of the separations of these ring pairs reported here suggest that the radii of the α and γ rings, and perhaps the ε_1

Table 1 Predictions of the location of the β and δ rings and the α - β and γ - δ ring separations using known satellite pairs, assuming that the α and γ rings are determined by the perturbations of Miranda-Ariel

Ring	Satellite pair	p ($q=1$)	Ring radius (km)	Ring separations Calc. (km)	Obs. (km)
β	Ariel-Umbriel	19	46,245	1,359	α - β 953
	Ariel-Oberon	9	46,724	1,838	
δ	Miranda-Umbriel	5	49,361	1,609	γ - δ 676
	Miranda-Titania	4	48,937	1,185	
	Ariel-Umbriel	17	49,347	1,595	
	Ariel-Oberon	8	50,041	2,289	

The resonances of Umbriel-Titania and Titania-Oberon have not been included as the resonance strengths for these perturbations as given by Goldreich and Nicholson⁶ are extremely weak.

and ε_2 components as reported by Elliott *et al.*¹, are governed by the resonances of Miranda and Ariel, with consistent further application of resonance theory to the location of the β and δ rings requiring the presence of an additional hypothetical satellite moving around Uranus. Without this hypothesis the observed ring system seems to be outside the scope of resonance theory alone.

Although resonance theory can be expected to predict the mean ring radii their widths are a more critical calculation and the predicted widths are probably too small, perhaps by an order of magnitude or more. Nevertheless, it is interesting to examine the consequences of possible resonance effects on the ring system and to investigate whether the mean location of the rings might coincide with resonance orbits. If the resonance orbits generated by the known satellites in the vicinity of the β and δ rings (collected in Table 1) are compared with the resonance orbits generated by the satellite pair Miranda-Ariel for the α and γ rings (given in Table 3) it is seen, in Table 1, that the derived ring separations α - β and γ - δ give virtually no agreement with the observed separations. Further, all the resonances given in Table 1 are much weaker than those which might govern the α , γ and ε rings⁶.

My calculations suggested that the β and δ rings might be determined by an additional source of resonance, arising from the combined action of Miranda and an inner undiscovered satellite (called for convenience Uranus VI). Subsequently Ip⁷ indicated a satellite orbit (radius $\sim 103,000$ km). Here I present evidence that to obtain agreement between the observed and calculated ring patterns, arising as a result of the resonances, the presence of an undiscovered inner satellite must be invoked.

Conditions for a three-orbit resonance, involving a ring particle and two satellites, have been described by Dermott⁸. For two satellites, with mean motion n_1 for the inner satellite and n_2 for the outer satellite, the mean motion n of a ring particle is obtained by solving the equation

$$qn - (p+q)n_1 + pn_2 = 0 \quad (1)$$

where for resonance p and q take integer values only. The strongest perturbations on the ring particle are associated with

Table 2 Calculated ring separations based on resonance orbits of the satellite pairs Uranus VI-Miranda (β and δ rings) and Miranda-Ariel (α and γ rings) compared with other observational data^{1,9}

Ring separation	Calc. (km)	Obs. (km) Elliot <i>et al.</i> ¹	Marsden ⁹
α - β	947	926	953
β - γ	1,919	1,920	1,944
γ - δ	672	672	676

small integer values of p and q , this condition gives rise to the most frequent conjunctions of the two satellites with the ring particle. For the calculations here $q=1$, and p is assigned various integral values. If a value of p is chosen to conform with a given ring, say δ , the mean motion of the unknown satellite (n_1) can be determined using equation (1) and its orbital radius deduced from Kepler's third law. This value of p then determines the integer sequence for the series calculation of other resonance orbits in the system. In this way we see that the two parameters p and n_1 (n_2 being determined by the orbit of the outer known satellite) will not determine the radii of both the β and δ rings automatically; only a particular choice of p for the two satellites concerned will achieve this.

To determine the radii of the β and δ rings, I assume that the perturbing effects of Miranda and Uranus VI are involved. On this basis it is found that for the β ring $q=1$ and $p=9$, while

for the δ ring $q=1$ and $p=8$ where n_1 is associated with Uranus VI moving in a circular orbit of radius 105,221 km (period 1.02428 d) coplanar with the rings, and n_2 refers to Miranda. The ring separations α - β and γ - δ that result from this choice are given in Table 2 with the calculated ring radii based on these parameters being included in Table 3. Other values of p , using the δ ring radius as a reference point to determine alternative orbits of the hypothetical satellite, give little agreement for the separation of the α - β ring pair. For example, setting the separation γ - δ at 670 km leads to calculated separations for the α and β rings of 645 km, with $p=8$ for the β ring, and 1,189 km with $p=10$ for the β ring, whereas the observed separation α - β is ~ 950 km.

The combined perturbations of known satellite pairs and satellite pairs involving the hypothetical Uranus VI give the parameters for the whole ring system, including the recently discovered κ , l , θ and η rings, as shown in Table 3. These additional rings were observed by E. Persson, P. Nicholson, K. Matthews, P. Goldreich and G. Neugebauer during an occultation on 10 April 1978 and reported by Marsden⁹. The most recent results also suggest that the ϵ ring is indeed complete and substantial. If this is so it could be consistent with the elliptical ring suggested by Lucke¹⁰, with the semi-major axis being determined by the 4:1 resonance of Miranda (radius 51,758 km). This perturbation has been shown⁶ to be the

Table 3 Predicted ring radii based on the resonances of the known satellites and the hypothetical Uranus VI compared with the observed radii given by Marsden⁹

Ring	Radius (km) Obs.	Radius (km) Calc.	Error (km) Obs. - Calc.	Satellite interaction*	$p(q=1)$
κ_1^+	42,029	41,979	+110	VI-Ariel	5
κ_2^+	42,148	41,983	+106	Miranda-Oberon	5
ϵ_1	42,394	42,397	-48	Ariel-Umbriel	22
ϵ_2	42,304	42,414	-65	Miranda-Ariel	10
θ_1	42,660	42,676	+2	Ariel-Titania	12
θ_2	42,696				
α	44,835	44,868	-33	Ariel-Umbriel	20
		44,886	-51	Miranda-Ariel	9
		44,880	-45	Miranda-Umbriel	6
β	45,788	45,833	-45	VI-Miranda	9
η_1	47,290	47,288	+2	Miranda-Oberon	4
η_2	47,289				
γ	47,732	47,733	-1	Ariel-Umbriel	18
		47,752	-20	Miranda-Ariel	8
δ	48,408	48,424	-16	VI-Miranda	8
ϵ	ϵ_1^{9b} 50,848	50,526	+322	Ariel-Titania	9
		50,583	+265	Uranus VI	(3:1)‡
	ϵ_2^{9a} 51,030	51,125	-95	Miranda-Ariel	7
	ϵ_2^{9b} 51,402	51,415	-13	VI-Miranda	7
	ϵ_1^{9a} 51,697	51,758	-61	Miranda	(4:1)‡

In the case of the ϵ ring the superscripts 9a and 9b refer to the occultations of 10 March 1977 and 10 April 1978 respectively.

*Resonance strengths for interactions involving known satellites are given by Goldreich and Nicholson⁶.

†The κ ring is reported by Marsden⁹ as being uncertain.

‡Resonance involving the ring particle and a single satellite moving in an orbit which is elliptical and, or, inclined to the ring plane.

Table 4 Satellite pair resonances in the vicinity of the hypothetical satellite Uranus VI

Satellite pair	$p(q=1)$	Resonance radius (km)
Miranda-Ariel	1	102,315
Ariel-Titania	2	106,368
Ariel-Umbriel	4	102,287
Umbriel-Titania	6	103,559
Titania-Oberon	21	105,906

strongest perturbation on the entire ring system and might, therefore, be expected to be associated with a major ring feature.

If Uranus VI accreted from a ring, or rings, of material similar to the present ring system around the planet, then the presence of this satellite is further suggested by the resonance effects of the other satellites in the uranian system. The orbital radius inferred for Uranus VI is in the vicinity of the resonances (having $q=1$) produced by the other satellite pairs listed in Table 4. The formation of a satellite having an orbital radius of around 103,000 km seems reasonable (especially as the entire satellite system of Uranus has a resonant or near-resonant structure) with the orbital radius of Uranus VI subsequently evolving, as a result of tidal interaction with Uranus, to the value as suggested by the present ring locations. A further point of interest is that if Uranus VI formed at a radius corresponding exactly to the $q=1, p=1$ resonance of Miranda-Ariel (period 0.9821 d), then the series of rings generated by the Uranus VI-Miranda resonances having $q=1$ and $p=8,7,6$ would have exactly the same radii as the present α and γ rings and the outer parts of the elliptical ϵ ring. This overlap could then provide a much stronger mechanism for the trapping of particles during the early stages of the formation of the ring system.

The orbital parameters for the known satellites used in the calculations were obtained from standard tables¹¹. In order to have escaped detection the inferred satellite must be a faint object (18-20 mag), and assuming an albedo similar to that of Miranda its maximum diameter could be of the order of 100 km. It should, however, be possible to detect this satellite with a large telescope employing photography in an absorption band of the uranian atmosphere.

The suggested introduction of Uranus VI into the satellite system of Uranus permits conventional resonance theory to give a reasonable quantitative account of the ring locations. The requirement of an additional satellite is valid only if resonance theory is involved, and it will not follow automatically if other, perhaps more subtle, mechanisms play a part in determining the ring locations. Finally, as suggested by Aksnes⁵, it may not be beyond the bounds of possibility that the rings are simply transitory features being found fortuitously in the vicinity of the satellite resonance orbits.

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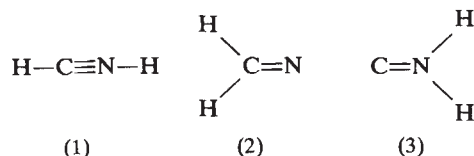
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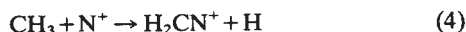
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Role of different isomers of the H_2CN^+ ion in the formation of interstellar HCN and HNC

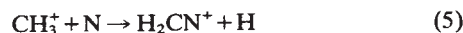
THE H_2CN^+ molecular ion is generally agreed¹⁻⁴ to have a critical role in the formation of interstellar HCN and HNC. The obvious problem that H_2CN^+ has not yet been identified in interstellar space is complicated by considerable uncertainty due to the possible existence of three distinct isomers of H_2CN^+ , with nuclear arrangements



Brown⁴ stated that although the third isomer had been completely neglected in previous discussion, it is likely to have a crucial role in the formation of HNC. Brown also notes that the linear isomer, which is isoelectronic with acetylene and thus expected to lie lowest of all the isomers of H_2CN^+ , may play no part at all in the formation of either HCN or HNC. This is from the hypothesis that the sequence thought most likely to lead to HCN is



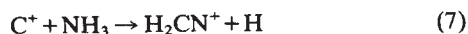
or



followed by dissociative recombination



Brown argues that the formation of the more stable linear isomer (1) would require the migration of a hydrogen atom from carbon to nitrogen. As this migration is expected to be very costly energetically, the H_2CN^+ on the right hand side of reactions (4) and (5) should be the isomer (2) above. Similarly, he argues that the sequence leading to HNC



should exclusively involve isomer (3). We report here theoretical studies designed to investigate the molecular structure of (3) and its energy relative to the previously studied³ isomers (1) and (2). In addition, because reactions (4), (5) and (7) must be exothermic or very nearly so if they play a part in the interstellar medium, this question has also been assessed.

The equilibrium geometry of isomer (3) was predicted using a double zeta (DZ) basis set⁵ and the self-consistent-field (SCF) approximation⁶. The theoretical structures of the three isomers are illustrated in Fig. 1. At this level of theory, gradient methods⁷ were used to demonstrate that each of the three isomers corresponds to a genuine relative minimum on the potential energy surface⁴. The transition states connecting (2) and (3) with the absolute minimum (1) were also determined theoretically and are included in Fig. 1.

The energetic relationships between (1), (2), (3) and the two transition states were then further delineated at two more nearly complete levels of theory. First, polarisation functions (d functions on C and N, p functions on H) were added at the SCF level, and second, this larger basis was used in conjunction with configuration interaction (CI) including all single and double excitations⁸. Table 1 summarises these energetic predictions

and the most remarkable result is that, contrary to chemical intuition (for example, H_2CO is the equilibrium geometry of formaldehyde rather than H_2OC), isomer (3) lies energetically well below isomer (2). To our knowledge the only serious previous suggestion of the existence of (3) is that of Brown⁴. Liddy, Freeman and McEwan⁹ have briefly noted the possibility of such a nuclear arrangement, but assume it to be unlikely on energetic grounds.

A second unexpected result is that when polarisation basis functions and correlation effects are taken into account, isomer (2) no longer seems to be a relative minimum on the potential energy surface. This conclusion, although highly probable based on the present theoretical treatment, cannot be put forward with complete confidence until a more complete search of the potential surface is carried out at our most sophisticated level of theory. In any case, there is at most a very small barrier for the rearrangement of isomer (2) to (1).

If the structure of H_2CN^+ is assumed to be the linear isomer (1), then the exothermicities of reactions (4), (5) and (7) can be determined from experimental thermochemical data¹⁰⁻¹³ to be exothermic by 209, 110 and 143 kcal, with each value uncertain by ~3 kcal. These large exothermicities are, of course, due to the formation of the CN triple bond.

Next, as suggested⁴ by mechanistic considerations, we should restrict the $\text{C}^+ + \text{NH}_3$ reaction (7) to have as its product the isomer (3) with both hydrogens attached to the nitrogen atom. This isomerically specific reaction, however, is still highly exothermic, by ~97 kcal. The remaining 97 kcal of internal energy is more than enough to pass over the 30 kcal barrier (transition state (5) in Fig. 1) leading to the linear absolute minimum of the H_2CN^+ potential surface.

Brown⁴ argues that the unimolecular rearrangements of isomer (3) "would be exceedingly slow at the temperatures prevailing in interstellar clouds;" this would be true if (3) were formed by a thermoneutral reaction. However, the large exothermicity accompanying the $\text{C}^+ + \text{NH}_3$ reaction will in this case lead very rapidly to the linear isomer, which in turn can produce either HCN or HNC by the dissociative recombination (6). Therefore, the reactions (4) and (5) need not necessarily be invoked to explain the interstellar formation of HCN. It is of interest, therefore, that the $\text{C}^+ + \text{NH}_3$ reaction is known to

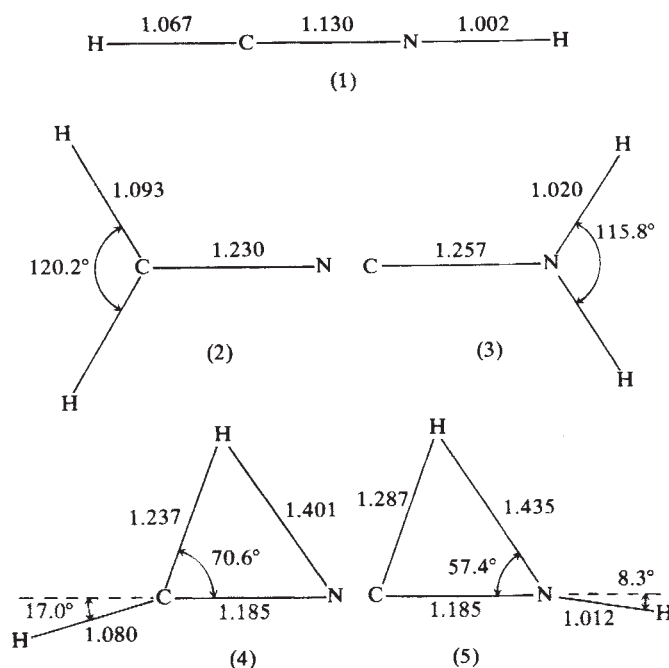


Fig. 1 Predicted structures for isomers (1), (2) and (3) and the transition states connecting them at the DZ SCF level of theory. Bond distances are in Å. Relative energies are: (1), 0.0; (2), 72; (3), 46; (4), 61; (5), 76 kcal mol⁻¹.

Table 1 Relative energies (in kcal mol⁻¹) of several points on the H₂CN⁺ potential energy surface

Structure (see Fig. 1)	Level of theory			
	DZ SCF	DZ CI	DZ+P SCF	DZ+P CI
(1)	0.0	0.0	0.0	0.0
(2)	69.6	57.3	65.4	71.6
(3)	41.4	31.4	40.0	46.0
(4)	80.0	78.3	61.5	60.9
(5)	93.7	88.4	79.1	75.6

Absolute energies of the linear isomer are -93.1290, -93.3119, -93.1758, and -93.4421 hartrees (where 1 hartree is 110.5×10^{-21} J).

occur rapidly¹⁴ (rate constant 2.3×10^{-9} cm³ s⁻¹), the CH₃⁺+N reaction is much slower¹⁵ (6.7×10^{-11} cm³ s), and the CH₃+N⁺ reaction has not been studied in the laboratory. (E. E. Ferguson, personal communication.)

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Note added in proof: It has now been confirmed that structure (2) is not a relative minimum on the DZ+P SCF, potential energy surface.

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Implications on unknown radio-activity of giant and dwarf haloes in Scandinavian rocks

GIANT haloes¹⁻³ attracted little attention until it seemed that those from Madagascar² might be associated with superheavy elements⁴. Even though this association was not confirmed⁵, this renewed interest has generated several additional suggestions⁶ for giant-halo origin which will be evaluated elsewhere (R.V.G. *et al.* in preparation). We report here some new data on the giant haloes found in certain Swedish biotites^{1,2} and the implications which these data furnish for a radioactive origin of the enigmatic dwarf haloes.

The majority of U and Th haloes in this Swedish biotite^{1,2} exhibit darkening which extends to the maximum halo radius (~38–40 µm for the Th halo). About 1% of haloes, however, have an inner bleached region which varies from ~2 to 25 µm in radius surrounding a highly radioactive inclusion. Generally, when the bleached region is small (≤ 6 –8 µm), no change is evident in the dimensions of the halo. However, in those haloes in which the bleached region is more intense and of larger radius (~15 µm), a somewhat weakly coloured diffuse ring is generally observed outside the normal U–Th halo boundary.

These are the giant haloes which, because they were earlier reported² to surround only dense Th haloes, were tentatively

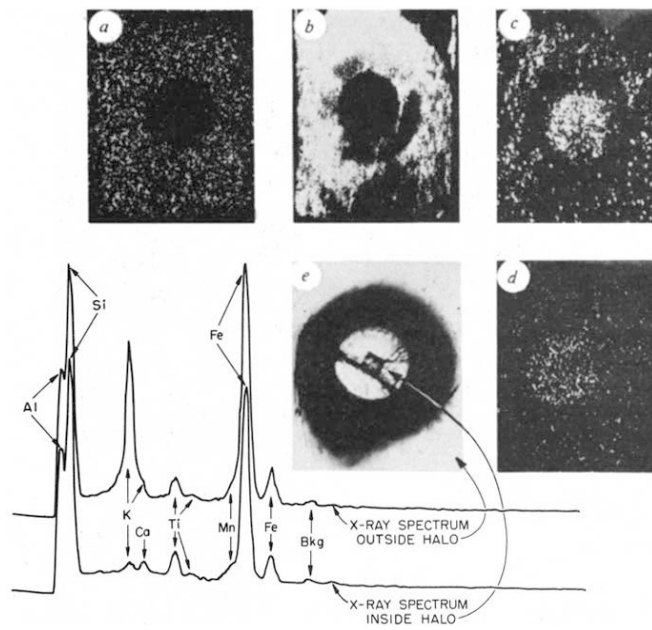


Fig. 1 Optical microscopic, electron microprobe and ion microprobe study of a giant halo in Swedish biotite. The giant halo in the optical photograph is ~47 µm in radius. The X-ray and ion probe maps are approximately the same magnification as the optical photo. a, K Kα X-ray map; b, ³⁹K secondary ion map; c, ⁴⁰Ca secondary ion map; d, Ca Kα X-ray map; e, optical photo.

attributed to the low abundance, high energy αs from ²¹²Po in the ²³²Th series. However, we now report that diffuse, abnormally large rings also surround dense U haloes in this biotite, and as there are no high energy αs of any significant abundance in the ²³⁸U chain, we therefore consider this hypothesis untenable. Instead, we are exploring the possibility that these bleached interior regions are somehow associated with the formation of the giant haloes.

Even though these giant haloes were found in Swedish granites obtained from the same location as the specimens Wiman¹ used, the giant haloes described here are different from those he reported. Our giant haloes surround U and/or Th rich inclusions and have diffuse boundaries which may vary from ~42 to ~55 µm in radius. In contrast, Wiman¹ reported giant haloes in biotite around zircon inclusions showing normal size inner rings and somewhat weak but rather sharply defined outer rings of 57 µm and more rarely of 67 µm.

To show the unusual characteristics of the giant halo we found in Swedish granitic biotites from Arnö and Rickaby, we give in Fig. 1 the combined results of applying optical microscopic, electron microprobe X-ray fluorescence and ion microprobe mass spectrometer techniques. In particular, Fig. 1 shows: a transmitted light optical photomicrograph of a single giant halo of radius ~47 µm; two secondary ion maps obtained (with the ion microprobe mass spectrometer) by rastering the halo region with a finely focused ¹⁶O⁻ beam and collecting the sputtered secondary ion signal at mass-to-charge ratios of 39 (³⁹K) and 40 (⁴⁰Ca); two X-ray maps obtained (using electron microprobe X-ray fluorescence) by rastering the same region with a 30-kV electron beam and collecting in sequence the K Kα and Ca Kα X rays; and the complete electron microprobe X-ray fluorescence spectra obtained by spot focusing the electron microprobe beam first on the mica completely outside the giant halo and then on the bright circular area inside it. In Fig. 1, the secondary ion and X-ray maps, as well as the contrasting X-ray spectra, show the region corresponding to the bright circular area in the optical photo (which differs slightly in magnification from the maps) is considerably diminished in K (by about a factor of 10) and slightly enhanced

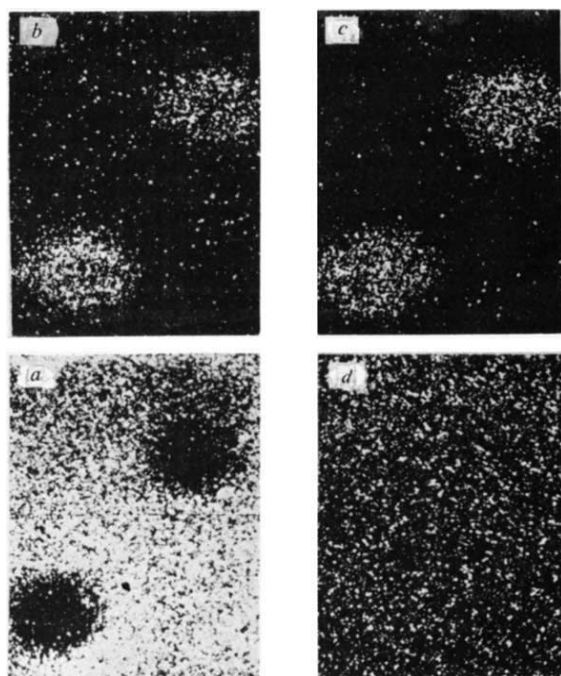


Fig. 2 Ion microprobe secondary ion maps of two dwarf haloes in Ytterby mica. *a*, ^{39}K ; *b*, ^{40}Ca ; *c*, ^{89}Y , and *d*, ^{28}Si . Dwarf haloes are $\sim 6\ \mu\text{m}$ in radius.

in Ca compared to the surrounding mica. Also, the secondary ion and X-ray maps in Fig. 1 reflect the composition of the halo region several micrometres above the central radioactive inclusion, the rectangular outline of which can be seen in the optical photo in Fig. 1. Certain mineralogical aspects relating to this phenomenon have been reported previously by Rimsaite⁹. These giant haloes probably did not result from diffusion of radioactivity into the mica because ion microprobe mass spectrometer studies showed U, Th and Pb were confined to the inclusion.

As the radius of the bright circular area in Fig. 1 approximates the range of the predominant lower energy α s of the U and Th series, we suggest that extreme radiation damage effects may have first produced a partial decomposition of the mica in this region with secondary effects then inducing the migration of K out of and Ca into this region. Note, for example, that the highly sensitive ion map of Ca in Fig. 1 shows a Ca depletion in the outer halo region, which is consistent with the idea that Ca has migrated inwards toward the central region.

As far as the formation of GH is concerned, the large K depletion in the central region may have been accompanied by depletion of other major elements during past epochs. If this happened, then for a certain period of time the total mass within this region may have reduced sufficiently to allow the highest normal energy α s of the Th and U series (8.73 MeV and 7.68 MeV respectively) to penetrate beyond the normal halo boundary because of having first passed through a region of lower density. Because of the large K depletion and slight Ca enrichment, this region has a slightly lower density than the adjacent mica. But unless O, which we have not as yet measured, is also depleted, the K (comprising only ~ 5 – 10 atomic % of the biotite) depletion alone would not be sufficient to permit normal energy α s to gain enhanced penetration of $\sim 15\ \mu\text{m}$.

Although there are unanswered questions about this phenomenon it seems that the bleached areas are high radiation damaged regions. This was very important to our studies of the dwarf haloes^{10,11}, the exact origin of which has remained a mystery for more than 50 years. Under the microscope, the dwarf haloes¹¹ exhibit the same type of bleached appearance as

is shown by the giant haloes in Fig. 1. Furthermore, in analysing the dwarf halo centres for the presence of some recognisable parent and/or daughter radionuclides, it became evident that the same type of K–Ca inversion phenomenon was showing up throughout the dwarf halo region.

Figure 2*a–d* shows several secondary ion maps obtained as an $^{16}\text{O}^-$ beam was rastered across two closely spaced dwarf haloes. The ion microprobe mass spectrometry ion maps in Fig. 2*a–d* reveal that these dwarf haloes were highly depleted in ^{39}K and enriched in ^{40}Ca and ^{89}Y with no change in ^{28}Si . The complete mass scans shown in Fig. 3*a, b*, contrasting the dwarf halo region (Fig. 3*a*) with the surrounding mica (Fig. 3*b*), reveal a corresponding depletion of ^{41}K , ^{85}Rb , and ^{87}Rb . Scanning electron microscopy X-ray fluorescence maps of many dwarf haloes also showed the depletion of K and enrichment of Ca throughout the dwarf halo, showing that the ion microprobe mass spectrometer results were not an artefact of the sputtering process. We consider the association of this K–Ca inversion phenomena with the dwarf haloes as additional evidence of a radioactive origin of the dwarf haloes.

In this context, it has been reported¹⁰ that dwarf haloes are rapidly etched by hydrofluoric acid. We now report that the halo periphery is more rapidly etched than the central part, which suggests the emission of a particle from the halo centre that caused greater damage (higher specific ionisation) near the end of its path. While this is a characteristic of α -particles, except for minute amounts of U found in some ion microprobe mass spectrometer scans, we have failed to find evidence that ^{147}Sm (ref. 11) or any other low energy rare earth α -emitters produced the dwarf haloes. That is, we searched for but found no significant concentrations of these nuclides in ion microprobe mass spectrometer scans of the halo centre. Instead we found that several rare earths were uniformly enriched (Fig. 3*a*) throughout the halo, compared to the surrounding mica (Fig. 3*b*). Even though Fig. 3*b* does not show any rare earths in the mica, other ion microprobe mass spectrometry and spark source mass studies showed that these elements do exist in the mica in very low abundance. We suggest that the mechanism for this enrichment is the same as that which caused the preferential transport of Ca (Fig. 2*b*) into the halo region (the ion maps of Ca and the rare earths are equal in extent). Note also the Y enrichment in the halo (Fig. 3*a*) compared to the mica (Fig. 3*b*).

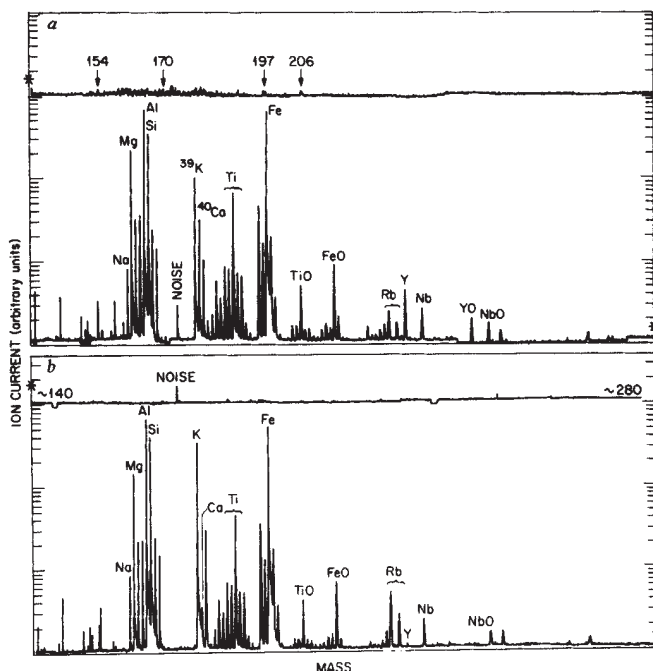


Fig. 3 Ion microprobe mass scans of (*a*) the dwarf halo and (*b*) the mica adjacent to the dwarf halo.

In conclusion, despite this new evidence for a radioactive origin of the dwarf haloes, more work is needed before the radionuclides which produced these haloes can be identified.

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Magnetic orientation of four-ball test specimens and the effect on total test time

THE rolling contact four ball test is a standard way of assessing the performance of lubricants in rolling contact¹. The test is well established and the effects of load and temperature on the results are well known^{2,3}. However, the effect of the anisotropic fibre structure of the balls is often overlooked despite the work of Anderson and Carter⁴ and Scott⁵. Part of the reason for this oversight is because until now there has not been a nondestructive method for orientating the balls. We describe here a simple, nondestructive way of orientating the top ball of a four-ball test and we show how this can be used to reduce the time needed to rank different lubricants according to their effect on the fatigue life.

The four-ball apparatus is simple. Three $\frac{1}{2}$ -inch diameter balls are driven in a race by a fourth, upper, ball which is held in a chuck, Fig. 1. The load between the balls is applied upwards by means of a lever arm and weights. The top ball has the most concentrated loading and eventually fatigues; a vibration sensor detects the pitting and stops the apparatus and timing mechanism. Occasionally, one of the bottom balls fails but this is not regarded as a valid result. The Institute of Petroleum method¹ recommends that 24 replicate runs are carried out to obtain reasonable statistics. The distribution function of the failure times is generally skewed but not sufficiently for gaussian statistics to be invalid (Kolmogorov-Smirnov test) and lubricants can be rated by the mean fatigue life.

Balls used in the four-ball test are typical of those manufactured for nonspecialised applications and are formed cold from a drawn billet of EN31 steel (1% C, 1.5% Cr) ground, lapped and polished and hardened to about Rockwell 66. The drawn

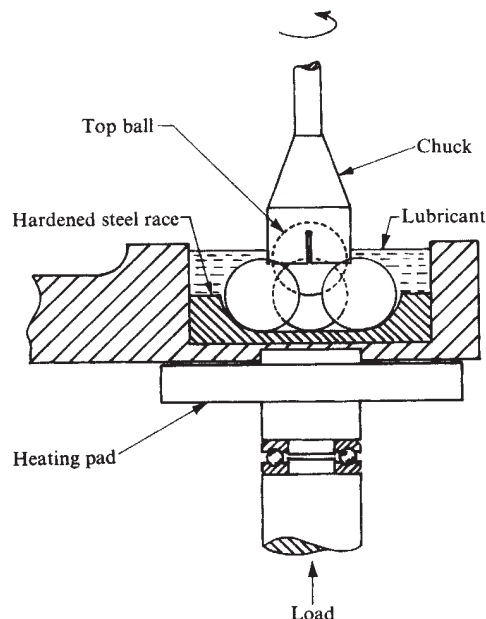


Fig. 1 The four-ball apparatus.

billet is not crystallographically isotropic and has a fibre structure along its length, this results in the balls having two polar regions and an equatorial band where the fibre is perpendicular to the surface. These regions are revealed by etching the ball for two minutes in boiling, concentrated HCl. The size of the polar regions in relation to the size of the ball and the width and diameter of the wear track means that there is a large probability (~ 0.88) of a track crossing a pole. Because the fatigue life is shorter on the poles^{4,5} most failures occur in these regions even if only a small proportion of the track crosses a pole.

Because of the structure of the balls we assumed that they would take up a preferred orientation in a magnetic field with the fibre structure lining up with the lines of force. The orientating force is very weak and the ball must be supported on a

Table 1 Fatigue lives of orientated and unorientated balls

Orientated balls		Unorientated balls	
Time to failure (min)	Position of pit	Time to failure (min)	Position of pit
67	E	30	P
93	E	36	P
99	OR	39	P
100	OR	40	P
103	OR	43	P
107	OR	47	P
115	OR	56	P
117	OR	58	P
123	OR	65	P
126	OR	69	P
136	OR	70	P
140	OR	72	E
140	OR	87	OR
155	OR	92	OR
167	OR	107	OR
181	OR	108	OR
184	OR	110	P
Mean life (min)	127	66	
Standard deviation (min)	32	26	

Position of the pit is indicated by: P, polar; E, equatorial; OR, other region.

Table 2 Number of tests and total testing time required to detect that oil B is better than oil A at the 90% level

	Oil A		Oil B		No. of tests for each oil	Total time (min)
	Mean life (min)	σ	Mean life (min)	σ		
Orientated	127	32	152	38	12	3,348
Unorientated	66	26	79	31	27	3,915

The total running time is taken as the product of the number of tests and the sum of the mean lives.

jet of air if it is to have any effect. With the apparatus shown in Fig. 2 the ball spins freely in the air jet with zero field but as the field is increased it becomes stationary in the orientation indicated in Fig. 2. The ball is then marked with a small spot of paint and removed. Balls direct from the manufacturer are already weakly magnetic and must be de-gaussed before they are orientated. Using this method we have been able to conduct replicate tests where none of the wear tracks cross a pole.

Two sets of 17 balls were tested at 2,000 r.p.m. with a load of 540 kg at 80°C, using a synthetic ester lubricating oil. One set of balls was orientated so that the wear track did not cross a polar region; balls from the other set were placed in the apparatus with a random orientation. After the top ball had failed it was removed and etched so that the position of the pit could be determined.

The fatigue lives, the position of the pit, and the mean and standard deviation of the fatigue lives are shown in Table 1. The decrease in fatigue life on the poles agrees with earlier work^{4,5} and at first sight there seems to be no reason for orientating the balls. However, if we calculate the number of tests needed to establish a significant difference between the effect of two different lubricants on the fatigue lives of orientated and unorientated balls the benefit of orientation is readily seen.

The usual method of determining the significance of the difference between two sets of measurements having means of x_1 and x_2 and standard deviations σ_1 and σ_2 is the t test. If the number, n , of measurements in each set is the same, then

$$t = (x_1 - x_2) / [(\sigma_1^2 + \sigma_2^2)/n]^{1/2} \quad (1)$$

We need to know the number of tests required to establish a significant difference between two oils; rearranging equation (1) we have

$$n = t^2(\sigma_1^2 + \sigma_2^2) / (x_1 - x_2)^2 \quad (2)$$

As t is a function of n , equation (2) has to be solved by an iterative process. Suppose we have two oils, A and B, one of which gives 20% better fatigue lives than the other. Then the

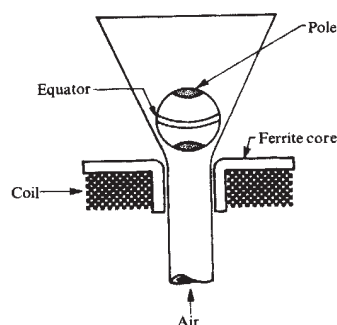


Fig. 2 Apparatus for orientating the balls; the magnetic field required to hold the ball in the indicated position is ≈ 500 A turns.

mean fatigue life and standard deviation would be increased by 20%. Table 2 shows the number of tests required to establish a significant difference between the oils at the 90% level and gives the total testing time required. Clearly, the time required to establish a significant difference between the two oils is less when the balls are orientated.

As the predicted number of tests is not the same as the number carried out, our value of the standard deviation is only an estimate. However, because of the large scatter in fatigue lives an increase in the number of tests from 17 to 27 rarely improves the standard deviation and it is worthwhile orientating the top ball of a four-ball test so that the wear track does not cross a polar region, as it can result in a considerable saving of time when ranking lubricants by their rolling contact fatigue performance. We also believe that this simple method of orientating balls will come to play an important part in the study of lubricant-fibre structure interactions which are of interest in the development of traction drives.

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Uranium-series dating of insular phosphorite from Ebon atoll, Micronesia

THE uranium-series method of absolute age determination has been widely applied to the radiometric geochronology of fossil coral reefs and associated problems of palaeoclimatology¹⁻³. Attempts have also been made to extend this method to marine phosphorites, leading to a better definition of the environmental conditions prevailing at the time of their formation⁴⁻⁸. Although the occurrence of phosphorite deposits on many islands in the central Pacific and Indian Ocean is well documented⁹, no absolute ages for any of these deposits have been available. This is regrettable because the widely accepted hypothesis⁹ for the origin of these insular phosphorites by chemical interaction of bird guano with the underlying reef limestones is based largely on circumstantial rather than direct evidence. We report here the first attempt to determine the age of both the apatite component as well as that of the associated coral material in a phosphorite deposit from Ebon atoll in Micronesia, in an effort to explore the feasibility of dating insular phosphorite deposits by the uranium series method and thus to relate the time of phosphate deposition to known environmental conditions in a given area.

The deposit in question has been briefly described by Hutchinson⁹, and is typical of small phosphorite deposits on several low-lying atolls in the Marshall Islands¹⁰. The sample on which this study is based was taken from a low cliff about 1.5 m above low tide level immediately behind the present beach on the seawards side of Ebon, the principal island of the atoll. The rock consists of coarse, pebble-size reef rubble, in a fine-grained carbonate matrix (Fig. 1a). Individual carbonate pebbles, mostly coral fragments, are invariably lined with light to dark brown phosphatic material up to 2 mm thick.

Microscopic examination (Fig. 1b) reveals that the phosphatic material has a laminate and often botryoidal structure with alternating light and dark bands coating carbonate pebbles

and filling voids within the carbonate framework. The phosphate seems to have formed as a result of direct precipitation on the surfaces of carbonate grains, rather than by replacement of the carbonate, although the latter process has also been shown to occur in similar phosphorite deposits on the islands of Remire in the Indian Ocean¹¹.

X-Ray diffraction analysis of the phosphatic material revealed the presence of apatite (carbonate hydroxy fluorapatite) as the sole component. The apatite peaks were quite broad and diffuse compared with those in seafloor phosphorites¹², probably due to extremely small grain size and poor crystallinity.

The composition of the phosphorite was determined along a transect extending from the apatite layer into the carbonate framework, using microprobe techniques. Figure 1c shows the location of five 'spots' analysed in an open-faced thin section. The results of these analyses are reported in Table 1. In addition to the elements shown, we also looked for Si, Al, Fe and several rare earths, but they were below the limit of detection in every case. The distribution of U in the phosphorite was revealed by neutron-induced fission tracks registered in Lexan plastic films (Fig. 1d). Note that in addition to the phosphorus, the elements Cl, F, Na, Mg and U are enriched in the phosphatic material relative to the carbonate. The overall chemical composition of the phosphate is similar to that of seafloor phosphorites, except for the F and U contents which are lower in the insular phosphorite.

The origin of F and U in insular phosphorites, even on high islands such as Christmas Island, Nauru and Makatea, is puzzling, as neither bird guano nor coral limestone contain sufficient amounts of these elements to be acceptable sources, and their derivation from seawater, either through seawater spray, or more directly at a time when these islands were partly submerged, has been suggested¹³.

The source and pathways of U are of particular interest in the present study, as it is relevant to the discussion of uranium series ages below. It would be tempting to suggest that the phosphate on Ebon island was deposited at a time when the sea level was somewhat higher than now. However, independent evidence for a slightly higher sea level stand in postglacial time at Ebon atoll and elsewhere in the Central Pacific is at best ambiguous¹⁴. As the phosphorite deposit lies within 1.5 m of low tide level, introduction of U and other elements such as Cl and F from seawater through seawater spray is an alternative.

To obtain meaningful ages for both the apatite phase and the coral component of the rock, the apatite rinds were carefully removed from several coral pebbles with a dentist's cutting wheel, and analysed separately. The results are shown in Table 2.

The principles and assumptions underlying the uranium series dating technique as applied to apatite have been dis-

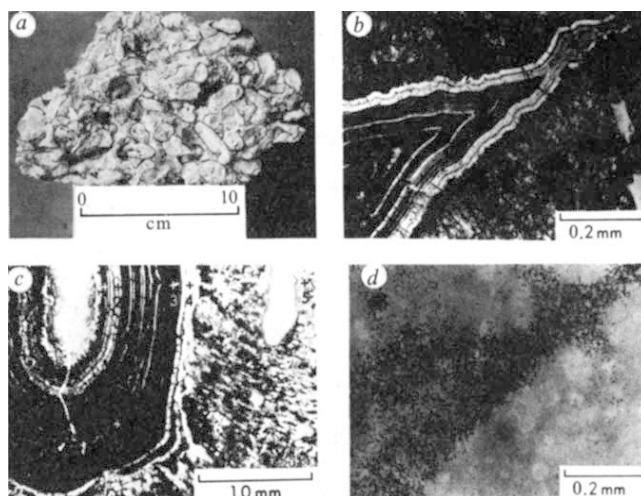


Fig. 1 a, Close view of hand specimen of phosphatic deposit on Ebon Island; b, microscopic view of laminar phosphatic material filling the void between carbonate pebbles; c, thin section view of Ebon Island phosphorite showing location of microprobe 'spots'. Annotations correspond to analyses 1-5 reported in Table 1; d, photomicrograph of Lexan plastic fission track detector showing distribution of uranium in same area as b. Each track represents the site of an atom of fissionable uranium which effectively maps the distribution of uranium within the area shown.

cussed elsewhere⁴⁻⁸. If we accept that (1) the uranium entered the apatite at the time of its formation; (2) the apatite was initially free of ²³⁰Th; (3) the apatite has remained a closed system with respect to uranium and thorium isotopes; and (4) the period of apatite formation was short compared with the absolute age to be measured, then the time of apatite formation is closely defined by the age calculated from the ²³⁰Th/²³⁴U ratio.

Assumptions (1) and (3) are difficult to evaluate on the basis of the available data. However, the uniform distribution of uranium within the phosphatic material shown by fission tracks (Fig. 1d) is consistent with a primary origin. Absence of ²³²Th strengthens assumption (2) as any non-radiogenic ²³⁰Th would be revealed by ²³²Th in the material. With respect to our problem, it is assumption (4) which requires discussion. Although an approximate upper limit for the onset of phosphatisation of the reef limestone is given by the age of the coral fragments associated with this deposit (about 5,000 yr) the actual duration of apatite growth is less well defined. If we assume that the deposition rate of apatite was linear with time, and that apatite growth has continued up to the present, we would expect a mean ²³⁰Th/²³⁴U age of 2,500 yr for the entire apatite rind which is significantly lower than our measured age of 4,000 yr. The estimate of 5,000 yr for the onset of phosphatisation is probably high, as we do not know the time interval between coral growth, reef erosion, emplacement of the coral rubble and the onset of apatite deposition in the pore space of the rubble. Furthermore, our ²³⁰Th/²³⁴U age of 5,000 yr for the coral is not very accurate, as shown by the large counting error. Radiocarbon ages of corals obtained from flat topped rubble ridges on Ebon Island range from 2,800 to 3,400 yr suggesting that the true age for the coral substrate may be less than 5,000 yr. Hence, we propose that the time of apatite formation is closely defined by the measured apatite age of 4,000 yr, and that the deposition of phosphate has ceased.

This interpretation raises an intriguing question: was phosphorite formation on Ebon atoll restricted to an episode in the late Quaternary when the environmental conditions were more favourable for the genesis of insular phosphorite deposits than at present? Hutchinson⁹ considered that high organic productivity in the ocean, coupled with low rainfall, were essential prerequisites for the net accumulation of bird guano, and hence for the genesis of phosphorites on islands, the former to ensure

Table 1 Microprobe spot analysis of a portion of the phosphate rocks used in this study

	1	2	3	4	5	Peru-Chile*
MgO	1.86	1.43	1.33	1.79	0.19	1.19
CaO	49.68	47.49	46.12	46.94	54.80	43.49
Na ₂ O	0.59	0.96	1.02	0.68	0.33	1.00
P ₂ O ₅	29.78	37.49	36.35	34.53	<0.1	28.54
F	2.59	1.40	1.00	1.82	Tr	2.89
Cl	0.25	0.30	0.57	0.29	Tr	0.28
CaO/P ₂ O ₅	1.67	1.27	1.27	1.36	—	1.52
F/P ₂ O ₅	0.087	0.037	0.027	0.053	—	0.101

* Average of 33 microprobe analyses of sea floor phosphorites from off South America¹².

Compositional data in weight percent. Numbers 1-5 refer to annotations on Fig. 1c which shows locations of spots analysed. Note that spot 5 is located within the limestone portion of the sample. Analyses of phosphorites from the sea floor off South America are given for comparison.

Table 2 Uranium and thorium isotopic data, and radiometric ages

Mineralogy	U (p.p.m.)	Th (p.p.m.)	$^{234}\text{U}/^{238}\text{U}$	$^{230}\text{Th}/^{234}\text{U}$	Age (yr)
Apatite	59.4	<0.05	1.15 ± 0.02	0.035 ± 0.003	$4,000 \pm 300$
Coral (aragonite)	2.70	<0.05	1.11 ± 0.09	0.043 ± 0.007	$5,000 \pm 1,000$

Errors quoted are based on counting statistics ($\pm 1\sigma$)

an adequate supply of food for a large bird colony, and the latter to prevent dense vegetation competing with ground nesting guano birds for space as well as to prevent the guano from being washed away by frequent heavy rains. These conditions currently prevail along a narrow belt just south of the Equator in the central and eastern Pacific, a region of exceptionally low rainfall (less than $1,000 \text{ mm yr}^{-1}$ on the average^{9,15}) and increased organic productivity caused by the equatorial divergence, and where contemporary guano deposits on islands are common⁹. Ebon Island, at $4^{\circ}35' \text{ N}$, is located north of the equatorial divergence and lies within the equatorial rainfall belt. It is heavily vegetated, and the production of guano is restricted to a limited number of tree nesting birds.

A world-wide change in climate, or at least a shift in climatic belts in the central and eastern Pacific during the late Quaternary, has been proposed by Hutchinson⁹ to explain the distribution of insular phosphorite deposits which do not show a clear relationship with the present climatic zonation. He was, however, seriously limited by a lack of absolute ages for any of the phosphorite deposits, and hence could not tie his hypothesis to an absolute time scale. Could the time of phosphatisation on Ebon atoll be related to the 'climatic optimum' about 5,000 yr ago, when temperatures were somewhat higher than now in many parts of the world, and most likely accompanied by slight changes in the location of the major climatic belts¹⁵? Clearly, more absolute ages of insular phosphorite deposits in critical locations are required before this question can be answered. For the present, we submit that insular phosphorites are suitable for age determinations by uranium series methods thus providing a new tool for palaeo-oceanographic and palaeo-climatic investigations in the Quaternary.

The sample from Ebon Island was collected by H.H.V. during the Scripps Carmarsel expedition to Micronesia in 1967. The experimental work was carried out with support from the Australian Research Grants Commission, and the NSF.

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K–Ar and $^{40}\text{Ar}/^{39}\text{Ar}$ Age of the Laschamp geomagnetic polarity reversal

THE Laschamp polarity reversal¹ remains one of the best documented cases of a recent excursion of the Earth's magnetic field. Unlike lake sediments, the Laschamp volcanics have a stable TRM (thermo remanent magnetism) which is not susceptible to structural degradation or detrital inclination error. Some workers have claimed to have seen the Laschamp excursion in recent sediments², while others have been unable to establish a correlation between the Laschamp and sedimentary core samples^{3,4}. One of the main difficulties with such a correlation is the uncertainty in the age of the Laschamp and Olby lava flows which record the excursion. We have performed a new series of K–Ar and $^{40}\text{Ar}/^{39}\text{Ar}$ analyses which suggest that the Laschamp excursion is significantly older than the widely quoted age reported by Bonhommet and Zähringer¹.

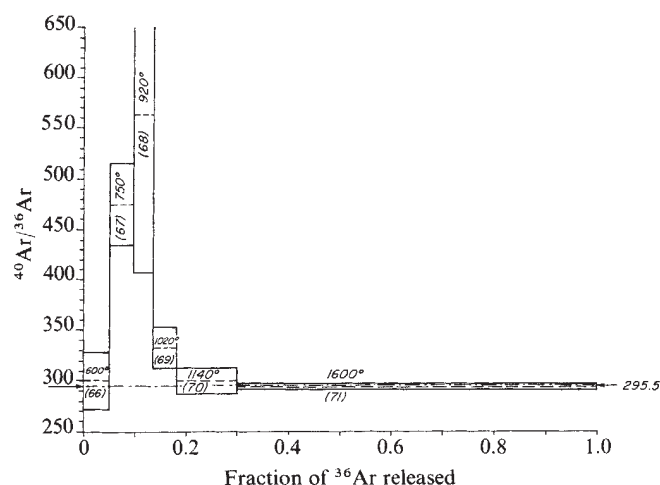


Fig. 1 $^{40}\text{Ar}/^{36}\text{Ar}$ release pattern for unirradiated Laschamp sample 444 (expt 66–71).

The results of Bonhommet and Zähringer¹ give an age between 8,000 and 20,000 yr BP. The lower limit was set by ^{14}C dating of baked trees found within a trachyte unit which overlies the Puy de Laschamp scoriae. As the scoriae and a nearby andesite flow have the reverse magnetisation, the ^{14}C date is a minimum age for the event. The upper limit was found by whole rock K–Ar dating of Laschamp and Olby flow samples. The Olby flow, which in the lower sections is reversely magnetised, is a basalt from the neighbouring Puy de Barme. Because of the small quantities of radiogenic ^{40}Ar measured, Bonhommet and Zähringer gave only upper limits for the age, with a final value of 20,000 yr BP.

Our results from conventional whole-rock analyses give a weighted average of $45,400 \pm 2,500$ yr (all error estimates in this paper are 1σ), significantly higher than the previous result of $<20,000$ yr obtained by Zähringer¹ (see Table 1). Our experiments were done with relatively large samples (roughly 10 g), while in the earlier K–Ar work, Zähringer¹ used samples weighing 1 g. Thus, we were measuring much larger volumes of gas. While this alone could account for an improvement in precision, it does not explain the systematic increase in age. Sample variability is also unlikely, as two of our samples were also analysed by Zähringer¹.

We believe that our results give consistently older ages because of a difference in correction for atmospheric (or initial) argon contamination. With samples this young and with

Table 1 Conventional K-Ar analyses on whole-rock chunks

Sample	Expt no.	%K	Mass (g)	Vol. atmos ^{40}Ar ($\times 10^{-8}$ ml NTP g $^{-1}$)	$^{40}\text{Ar}/^{36}\text{Ar}$	Age (kyr BP)
A1 (Olby)	16	1.722	13.94	5.60	313.9 $\pm$ 4.5	61.5 $\pm$ 13.0
	18		12.51	5.63	312.9 $\pm$ 2.4	60.3 $\pm$ 8.4
	22		11.62	6.89	307.8 $\pm$ 1.6	54.1 $\pm$ 8.4
	26		16.39	6.00	311.2 $\pm$ 1.6	46.2 $\pm$ 5.9
	27		12.21	5.27	310.2 $\pm$ 1.7	38.2 $\pm$ 5.3
	47		10.79	6.50	309.3 $\pm$ 3.3	44.2 $\pm$ 11.0
443 (Olby)	32	1.775	8.14	3.24	322.0 $\pm$ 3.8	40.9 $\pm$ 6.2
	39		3.87	3.68	307.7 $\pm$ 11.4	21.5 $\pm$ 20.0
444 (Laschamp)	33	1.905	4.23	4.37	315.2 $\pm$ 4.2	38.3 $\pm$ 8.5
	34		7.42	3.45	408.1 $\pm$ 4.5	173.0 $\pm$ 9.4
	106		9.55	7.26	311.4 $\pm$ 1.9	51.2 $\pm$ 8.1

All error estimates are 1 s.d. Weighted average of the above ages (excluding expt 34 which probably has excess ^{40}Ar) is 45,400 $\pm$ 2,500 yr. $\lambda_e = 0.584 \times 10^{-10} \text{ yr}^{-1}$; $\lambda_\beta = 4.72 \times 10^{-10} \text{ yr}^{-1}$; $^{40}\text{K} = 0.0119 \text{ atom \% of total K}$.

$^{40}\text{Ar}/^{36}\text{Ar}$ ratios this low, the age found is critically dependent on the assumed atmospheric $^{40}\text{Ar}/^{36}\text{Ar}$ ratio. Our ages were calculated using the standard atmospheric ratio of 295.5. This is a somewhat arbitrary value, as the initial argon contamination might have had an $^{40}\text{Ar}/^{36}\text{Ar}$ ratio either above or below 295.5. Independent evidence is necessary to justify the use of 295.5 as the initial argon contamination correction factor.

The atmospheric correction used by Zähringer¹ was based on the isotopic composition of argon from blank experiments. This is a valid correction if most of the ^{36}Ar detected in an actual experiment is from the system itself, or if, by chance, the system argon is identical in composition to the initial argon trapped within the sample. If neither case holds, then a fractionated system blank might shift the calculated age up or down.

Our original conventional K-Ar studies showed that the atmospheric contamination was derived from the sample, as measured atmospheric ^{40}Ar volumes were typically 20 times the volume of the system blank. Interestingly, the measured volumes of ^{36}Ar for our analyses were virtually the same per gram as those measured by Zähringer¹. Thus, the ^{36}Ar measured in his experiments was also probably sample-derived.

We also performed multiple temperature step heating experiments on the samples to find how strongly the atmospheric argon was retained. Figure 1 shows the results of such an analysis on sample 444. Note that most of the ^{36}Ar was

Table 2 Summarised $^{40}\text{Ar}/^{39}\text{Ar}$ results

Sample	Expt no.	High precision fraction* age (yr BP)	Integrated age (yr BP)
A1 (Olby)	127-133	46,700 $\pm$ 2,100	44,200 $\pm$ 14,800
	151-157	47,100 $\pm$ 6,100	43,100 $\pm$ 24,900
443 (Olby)	135-141	42,900 $\pm$ 9,000	53,500 $\pm$ 12,800
444 (Laschamp)	119-125	117,000 $\pm$ 4,000	119,000 $\pm$ 12,000
	144-150	60,100 $\pm$ 7,700	47,600 $\pm$ 16,300

All error estimates are 1 s.d. Weighted averages of the above ages (excluding expt 119-125 which probably has excess ^{40}Ar) are 47,400 $\pm$ 1,900 yr for the high precision fractions and 48,400 $\pm$ 7,900 yr for the integrated ages.

*The high precision fraction is that fraction near 800 °C with high $^{40}\text{Ar}/^{36}\text{Ar}$ ratio, large volume of ^{39}Ar , and low $^{37}\text{Ar}/^{39}\text{Ar}$ ratio.

released at temperatures above 1,100 °C, indicating that the bulk of the atmospheric contamination was within highly retentive minerals. Also, the argon released at the highest temperatures had almost exactly atmospheric isotopic composition. All of the samples studied showed this same pattern of release, so we feel that the use of 295.5 as the initial $^{40}\text{Ar}/^{36}\text{Ar}$ ratio is justified.

Another remarkable feature of Fig. 1 is the pulse of radiogenic ^{40}Ar which was released at temperatures around 800 °C. All of the samples studied had a significant enrichment in the $^{40}\text{Ar}/^{36}\text{Ar}$ ratio in this temperature range. The measured $^{40}\text{Ar}/^{36}\text{Ar}$ ratios ranged from about 330 to over 800, and the amount of radiogenic ^{40}Ar released around this temperature could account for the ages found with the conventional analyses.

The high $^{40}\text{Ar}/^{36}\text{Ar}$ ratios found at 800 °C encouraged us to try $^{40}\text{Ar}/^{39}\text{Ar}$ dating of these samples. The $^{40}\text{Ar}/^{39}\text{Ar}$ age spectrum for sample A1 is shown in Fig. 2. It is clear from this plot that, as with radiogenic ^{40}Ar , most of the ^{39}Ar (which is derived from ^{39}K) was released around 800 °C. The 800 °C fraction also gives an extremely precise age of 46,700 $\pm$ 2,100 yr. Figure 3 shows why the $^{40}\text{Ar}/^{36}\text{Ar}$ technique achieved results as precise, if not more precise, than conventional K-Ar analyses; this $^{40}\text{Ar}/^{36}\text{Ar}$ release pattern corresponds to the A1 age spectrum in Fig. 2. Here, as in Fig. 1, there is a high $^{40}\text{Ar}/^{36}\text{Ar}$ ratio at 800 °C. A large fraction of the ^{36}Ar is released at high temperatures, and the $^{40}\text{Ar}/^{36}\text{Ar}$ ratio at the highest temperatures is very close to 295.5. Thus, the very process of step heating has effectively isolated the radiogenic ^{40}Ar from the bulk of the contaminating atmospheric ^{40}Ar . Another advantage of step heating is that the age

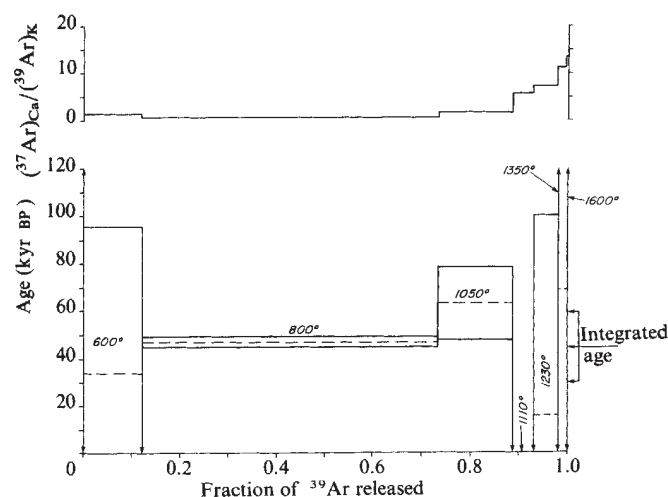


Fig. 2 $^{40}\text{Ar}/^{39}\text{Ar}$ age spectrum for Olby sample A1 (expt 127-133). The top graph shows the ratios of calcium derived ^{37}Ar to potassium derived ^{39}Ar .

deduced from around 800 °C is relatively insensitive to uncertainties concerning the initial $^{40}\text{Ar}/^{36}\text{Ar}$ ratio trapped within the rock. Even if the initial $^{40}\text{Ar}/^{36}\text{Ar}$ ratio was as high as 310, which is extremely unlikely from the $^{40}\text{Ar}/^{36}\text{Ar}$ release pattern, the age calculated from the 800 °C fraction would be reduced by only 12%, as the $^{40}\text{Ar}/^{36}\text{Ar}$ ratio of this fraction is over 410.

For each irradiated sample, there was a fraction near 800 °C with a high $^{40}\text{Ar}/^{36}\text{Ar}$ ratio, and a correspondingly high precision (see Table 2). The weighted average of the ages from these fractions is $47,400 \pm 1,900$ yr BP. This represents four analyses, two of A1 and one each of 443 and 444. It is interesting that the weighted average of the four integrated $^{40}\text{Ar}/^{39}\text{Ar}$ ages is $48,000 \pm 7,900$ yr BP (see Table 2). Comparing these values with the conventional age average of $45,400 \pm 2,500$, there is excellent agreement between our conventional ages and our $^{40}\text{Ar}/^{39}\text{Ar}$ ages.

We attempted to date finely crushed material but were unsuccessful due to an unacceptable laboratory-induced increase of the atmospheric argon contamination. Also, two analyses of 444, one irradiated and one conventional, gave anomalous ages over 100,000 yr BP. Bonhommet and Zähringer¹ also reported this phenomenon with sample 444, and we agree with their explanation that this sample probably contains small quantities of undegassed phenocrysts which are not present in every chunk. Despite such an occasional setback, the vast majority of analyses of chunks of all samples gave reproducible results.

It should be noted that the use of $^{40}\text{Ar}/^{39}\text{Ar}$ dating method, with step heating, on rocks of this age is unprecedented. These Laschamp analyses extend downwards the age of rocks to which $^{40}\text{Ar}/^{39}\text{Ar}$ step dating has been applied by a factor of >100. This dating technique shows considerable promise when used with young rocks because of its potential ability to separate radiogenic ^{40}Ar from atmospheric ^{40}Ar and to reduce ambiguities in initial $^{40}\text{Ar}/^{36}\text{Ar}$ ratios³.

It seems that the K-Ar evidence points to an age for the Laschamp excursion of about 47,000 yr BP and not an age under 20,000 yr BP. Thus, it is unlikely that the 12,000-yr-old lake sediment excursion², or the Mono Lake excursion³ could be correlated with the Laschamp reversal. One lake sediment excursion which possibly corresponds to the Laschamp is the 49,000-yr-old reversal in Lake Biwa, reported by Yaskawa *et al.*⁶. In any event, the search for the Laschamp excursion in lake sediments should be concentrated in sediments older than 20,000 yr BP, ideally covering an age range from about 30,000 to 60,000 yr BP.

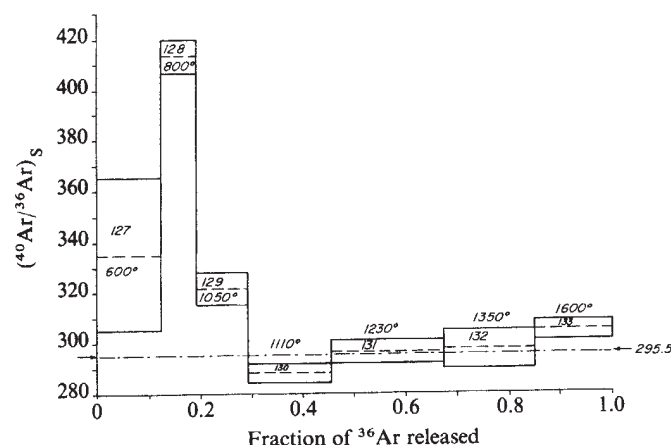


Fig. 3 $^{40}\text{Ar}/^{36}\text{Ar}$ release pattern for irradiated Olby sample A1 (expt 127–133) corrected for irradiation-induced isotope interference effects. Note the resemblance between this figure and Fig. 1.

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Iceberg calving from floating glaciers by a vibrating mechanism

OBSERVATIONS of Antarctic super tabular icebergs^{1–5}, which can exceed 100 km on a side, have shown that their origin can frequently be traced to previously existing super ice tongues, which are a class of massive seawards-extending ice shelves. Furthermore, a history of cyclic growth and calving can often be inferred^{2–4} or demonstrated^{6,7}. Because the calvings do not generally occur along a line coincident with the grounding line, where, under normal tidal flexure the stresses are greatest^{8,9}, and because multiple fracturing is observed^{1,2} we have sought a nontidal theory for ice-tongue fracture⁹. A mechanism for generating significant bending stresses at locations along an ice tongue far from the grounding zone is by vibration of the glacier in a mode higher than the fundamental¹⁰. In this report, we outline an approximate analysis of the modes of free vibration of a buoyant, elastic, tapering ice tongue floating in shallow water of variable depth.

Ocean wave energy in filtered form is transmitted through shelf ice^{11,12}. We consider that certain ocean waves of sufficient energy and suitable period are able to excite the corresponding mode of vibration of the glacier. In suitable conditions a resonant-like motion may occur. For high values of incident wave energy—such as those generated by local storms, and such that a high mode is excited—ice curvature, and hence the bending stress at a particular location of the glacier, may be sufficiently great, that, in a cyclic bending regime, fatigue failure may occur. Where these stresses coincide with zones already weakened by tidal flexure^{8,9}, reinforcement of the bending process may take place. In addition, a significant longitudinal tensile stress already exists in shelves more than ~200 m thick, because of plastic creep of the ice¹³. Therefore the additional stresses needed for total fracture may not be very high. Also, as a result of flow across the grounding line, plastic creep and bottom melting^{7,14} the ice tongue lengthens and thins, causing the vibrational characteristics to change with time. Thus, in effect, the glacier is able to sweep across a band of frequencies during its existence. After the last calving event, the probability of the next calving will evidently increase with time.

Recent observations⁵ indicate that in some cases super tabular icebergs may themselves trigger calvings of large ice tongues by direct impact. The total energy of a drifting super iceberg may reach 10^{20} erg, more than enough (by several orders of magnitude) to induce significant stresses in a large ice tongue.

However, not all documented ice tongue calvings seem to have originated in this way^{1,7}, and the origin of the first super tabular iceberg must be explained in such a scheme.

Here we take Erebus Glacier Tongue (lat 77° 42'S, long 166° 40'E) as a basis for the model for which an analysis is presented here. Although this feature is an order of magnitude smaller than the super ice tongues, the same mechanism is considered applicable on both scales. The glacier seems to exhibit a history of cyclic growth and calving⁷ and we suggest that the latter event might be explained by the mechanism proposed here. It is known that (1), crevasses exist on the surface of the glacier, (2) the lateral margins are characterised by edge cracks, (3) the surface contains a series of waves or 'rolls', (4) there is a temperature and density gradient with depth in the ice, and (5) plastic creep is taking place within the glacier. These features have not formally been accounted for because the difficulty of the solution would reach unreasonable proportions, and yet not alter the basic conclusions. A physically idealised ice tongue is shown in Fig. 1. The coordinate system and variables used are also shown. We wish to derive the natural frequencies of free vibration and corresponding modal shapes for this system. The assumptions made are that the plate is composed of a homogeneous, isotropic, isothermal, linear elastic solid. The present analysis may be improved by considering ice to have visco-elastic properties and by considering the forced vibration case. A corresponding analysis using visco-elastic theory would yield qualitatively similar results but would not alter our essential conclusions. Likewise, the discussion of stresses here is only approximate and not meant to imply a totally sufficient treatment. For periodic oscillations substantially less than ~ 1 min, the use of elastic theory is considered partly justified¹⁵. Because the expansive plastic creep of the ice tongue¹³ cannot be combined with the present formulation, it is thus considered separately, although evidently, the cyclic stresses may enhance the horizontal creep process¹⁶. We further assume that the usual conditions for thin plate theory to be valid, approximately apply here. The rotary inertia and shear effects are found to be small. We ignore ice-water skin drag effects.

Using thin plate theory, the fundamental equation¹⁷ relating bending moments to lateral loading, corresponding to a certain vertical plate deflection, $\omega(x)$, may be written as:

$$\frac{\partial^2 M_x}{\partial x^2} + \frac{\partial^2 M_y}{\partial y^2} - 2 \frac{\partial^2 M_{xy}}{\partial x \partial y} = 2\rho_i h \frac{\partial^2 \omega}{\partial t^2} + \rho_w g \omega + p \quad (1)$$

where

$$M_x = -D \left(\frac{\partial^2 \omega}{\partial x^2} + \nu \frac{\partial^2 \omega}{\partial y^2} \right)$$

$$M_y = -D \left(\frac{\partial^2 \omega}{\partial y^2} + \nu \frac{\partial^2 \omega}{\partial x^2} \right)$$

and

$$M_{xy} = -M_{yx} = D(1-\nu) \frac{\partial^2 \omega}{\partial x \partial y}$$

are, respectively, the bending moments in the longitudinal (x) direction, in the transverse (y) direction and the twisting moment. The flexural rigidity, D , of the plate is given by $D = 2E_i h^3 / 3(1-\nu^2)$ where E_i is the average dynamic modulus of elasticity of the ice, in bending, ν is Poisson's ratio and $h(x)$ is the half thickness of the slab (neutral axis assumed central). The terms on the right side of equation (1) are, respectively, the inertia term (ρ_i is the density of ice, t is time); the buoyancy term (ρ_w is the density of seawater, g the acceleration of gravity); and p is the deviation from hydrostatic pressure in the water under the plate.

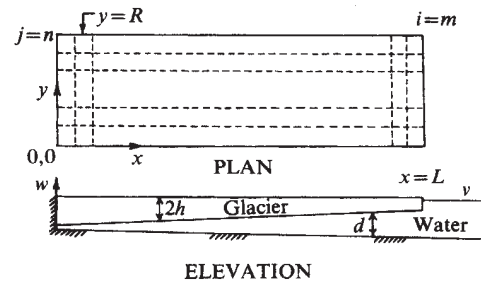


Fig. 1 Idealised model ice tongue of length L , width R and thickness $2h(x)$ floating in shallow water of depth $d(x, y)$. Grid (i, j) is for the finite difference scheme used to solve the equations given in the text. Values of $n = 6$ and $m = 47$ were used.

For the water layer, the equations of continuity and fluid motion are combined to give

$$\rho_w^{-1} \nabla \cdot (d \nabla p) = -\partial^2 \omega / \partial t^2 \quad (2)$$

where the operator $\nabla = (\partial/\partial x, \partial/\partial y)$ and $d = d(x, y)$ is the depth of water under the glacier.

The assumptions made in deriving equation (2) are that the water depth increases slowly with (x, y) , and that the water, of uniform density, is both incompressible and nonviscous. The flow is assumed laminar and uniform in a vertical column¹⁰.

The coupled equations (1) and (2) may be solved by the method of separation of variables by making the following transformations:

$$(M'_x, M'_y, M'_{xy}, \omega', p') = (M_x, M_y, M_{xy}, \omega, p) \sin \alpha t$$

where α is the frequency of the oscillation.

The boundary conditions (Fig. 1) are as follows:

$$\text{For } x = 0, \omega' = 0, \frac{\partial \omega'}{\partial x} = 0, \quad M'_x = \nu^{-1} M'_y = -D \frac{\partial^2 \omega'}{\partial x^2},$$

$$M'_{xy} = 0,$$

$$\text{and } \frac{\partial p'}{\partial x} = 0.$$

(In practice, this boundary may not necessarily be rigidly fixed. If so, then the bending moments at the grounding line will be less than those computed.)

$$\text{At } x = L, M'_x = 0, \quad M'_y \approx D\nu^{-1}(1-\nu^2) \frac{\partial^2 \omega'}{\partial x^2},$$

$$\frac{\partial M'_x}{\partial x} = 2 \frac{\partial M'_{xy}}{\partial y}, \quad M'_{xy} = D(1-\nu) \frac{\partial^2 \omega'}{\partial x \partial y}, \quad \text{and } p = 0$$

Similar boundary conditions exist on the other free edges defined by $y = 0, R$ for all x and t (Fig. 1).

Central finite difference formulae were then applied to the variables in order to convert the coupled system of boundary value problems into a generalised matrix eigenvalue problem which was solved iteratively to find the dominant values of α . Because of the large number of unknown variables, out-of-core gaussian elimination and back substitution routines were used and the eigenvalues were computed by the dominant root method.

To obtain specific deflection values for the plate we have transferred a finite amount of energy into the plate for each mode. The deflections needed to account for this amount of energy, given by:

$$\int_0^R \int_0^L \left[-\frac{1}{2} M_x \frac{\partial^2 \omega}{\partial x^2} - \frac{1}{2} M_y \frac{\partial^2 \omega}{\partial y^2} + \frac{1}{2} M_{xy} \frac{\partial^2 \omega}{\partial x \partial y} - \frac{1}{2} M_{yx} \frac{\partial^2 \omega}{\partial y \partial x} + \int_0^\omega \left(2\rho_i h \frac{\partial^2 \omega}{\partial t^2} + \rho_w g \omega + p \right) d\omega \right] dx dy$$

have then been determined for each mode.

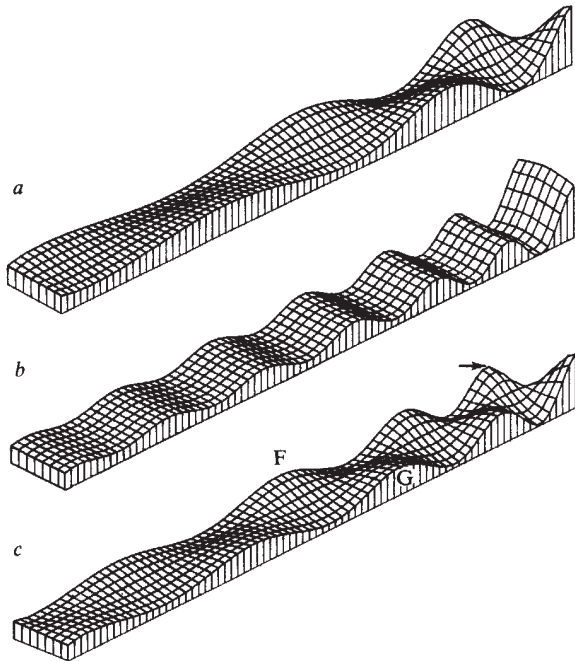


Fig. 2 Three-dimensional computer plots of the deflection of the surface of the model ice tongue for three modes. Length 14 km, width 1.5 km. *a*, period 24.2 s; *b*, period 20.2 s; *c*, period 16.0 s. This mode is considered to be the most likely mode at failure, since dominant oscillations of about this periodicity have been measured on the glacier⁷. This periodicity approximately coincides with the peak in the wave swell spectrum. Vertical scale exaggeration is $10^4:1$ with a maximum deflection of 10 cm. Left hand end is fixed, and taken as a clamped boundary in the computations. In practice, the boundary is probably partly flexible which would reduce the bending moments (and hence the stresses) as computed there. The deflection curve would also be modified mostly near the fixed end. The observed trace of the calving line⁷ is indicated by F-G.

Selected results are given in Fig. 2 which shows three-dimensional plots for an ice plate 14 km long, 1.5 km wide and with a fixed end thickness of 355 m tapering to 71 m at the free end. Water depth under the ice increases from 20 m at the fixed end to 233 m at the free end. These values approximately apply to the present Erebus Glacier Tongue. The approximate elastic bending stresses were computed from the corresponding bending moments in the usual way for a central neutral axis. Shear stresses turn out to be nearly an order of magnitude smaller than the bending stresses. We find that in order to produce a surface bending stress of about 0.05 MN m^{-2} on the crest of an antinode, as marked in Fig. 2c, a deflection there of about 5 cm is required. This would require a total energy input to the glacier of about 10^{14} erg . Although measurements under extreme conditions are not available, under normal conditions, shelf ice deflections are thought to be only a few per cent of the above deflection although for much thicker ice on confined ice shelves¹¹. Because crevasses exist down to a depth of at least 30 m the effective ice thickness is correspondingly less than we have taken and hence the corresponding bending stresses will be much higher especially towards the free end of the glacier. For example, where the actual ice thickness is 100 m the stresses for a given deflection are doubled by this effect. Also the existence of the surface 'rolls' would produce local deviations in the bending stresses.

Having used elastic theory to primarily determine modal shapes we only wish to qualitatively discuss bending stresses using elastic theory because of the complexity of the real system. The cyclic stresses, maximising at antinodes, will be superimposed on the expansive creep stress in the glacier, which for a 200 m ice thickness, is about 0.04 MN m^{-2} . For this

reason, the cyclic stresses may not have to be very high in order to just propagate one of the existing surface fractures which exist in profusion. Where principal antinodes coincide with secondary tidal bending zones^{8,9} reinforcement of stresses will occur. However, this effect diminishes rapidly away from the grounding line. The possible existence of basal cracks¹⁸ which have been filled with frozen seawater, would introduce another structural anisotropy that might accelerate failure of the ice if such a 'crack' aligned with an antinode of vibration. We suggest that the geometry of the fracture would be determined by the geometry of the trace of the antinode in the region of failure (Fig. 2F, G).

Thus it seems possible that the proposed vibration mechanism, although normally giving rise to weak bending stresses, may, under high incident wave energy, cause calving. Other phenomena, discussed above, probably play an integral part of the process of fracture, but the trace of antinode crests of the modal curve probably largely determines the position and shape of the main fracture(s) and hence the size of the resulting iceberg(s).

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Foxing, a fungal infection of paper

IRREGULAR yellowish-brown patches, or 'foxing', often occur on old books and prints, but their cause is nevertheless uncertain. One possibility is fungal infection¹⁻¹¹, suggested by the destructive pigmented lesions seen on mildewed cloth¹²; but foxed paper is almost invariably intact and never looks mouldy to the naked eye. Chemical causes have therefore been suggested, such as dampness¹³ affecting impurities in the paper¹⁴, connected in some way with either poor bleaching¹⁵ by chlorine¹⁶, or poor washing¹⁷, or the accumulation of visible iron salts in the lesions^{2,7,8,11}. If the cause were indeed fungal infection, the most reliable method of proof should be microscopy rather than culture, considering that fungal spores are ubiquitous and to be expected in virtually any sample of paper. Moreover, with these unusual lesions that may have taken half a century to develop, a positive culture might merely reflect a fast-growing contaminant and not the true cause. The isolations reported^{1,3-5} are therefore difficult to assess, added to which some came from paper that was frankly mouldy¹. We find that fluorescence microscopy invariably reveals fungal mycelium in foxed lesions whose properties can be explained by fungal growth occurring in an unfavourable environment.

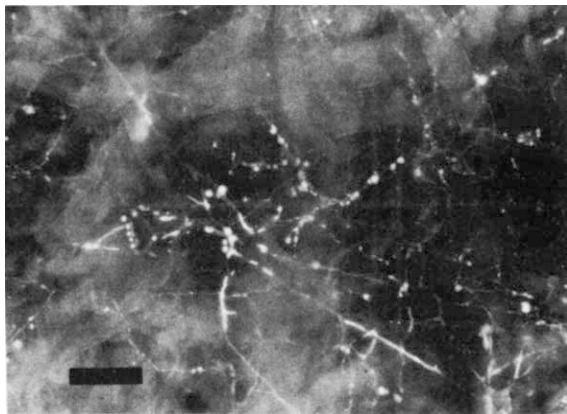
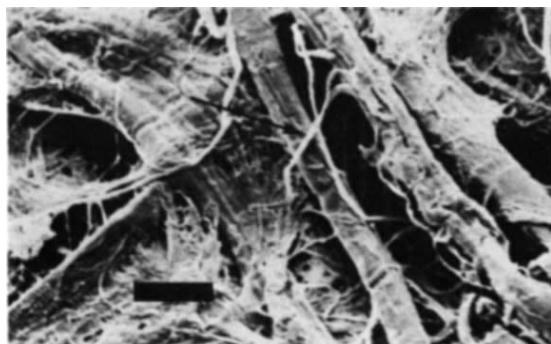


Fig. 1 Foxed lesion viewed by fluorescence microscopy, showing an above-average concentration of hyphae. Pieces of paper were fixed for 1 min in Carnoy (10 ml of glacial acetic acid; 30 ml of chloroform; 60 ml of absolute ethanol), washed for 1 min in 70% (v/v) ethanol; and rinsed and then mounted wet in 20% (w/v) KOH containing acridine orange freshly added to 0.01% (w/v). Optics: HBO 200 W/4 ultraviolet source; Zeiss model II vertical illuminator with Zeiss $\times 16$ Neofluor objective; Zeiss exciter filters BG 38/2.5 and BG 3, and barrier filters, 50 and 44. The bar represents 50 μm . Conventional acridine orange staining could sometimes be used but often stained the cellulose very heavily, while the hyphae faded in 5–10 min, even without exposure to ultraviolet light.

Foxed paper was taken from eleven books published between 1842 and 1919 and from tissue paper inserts dating from 1850–1919. Low-power microscopy ($\times 20$ – 40) confirmed that foxed areas did not appear mouldy. Using high power fluorescence microscopy ($\times 160$ – $1,000$), excellent contrast was obtained between the mycelium and the background of cellulose fibres by using a stain adapted from dermatology¹⁸ (Fig. 1). Foxed areas invariably showed fungal hyphae weaving around, but not within, individual cellulose fibres, whereas the surrounding normal paper was almost free of mycelium. Hyphae usually stood out as bright lime-green filaments studded with bright green dots (presumably sporangia; Fig. 1) although, occasionally, they were a faint dull green and presumably empty and dead. The background of cellulose was a dull olive-green with some diffusely brighter areas. The scarcity and patchy distribution of hyphae within each lesion was particularly striking^{3,5} and was confirmed by scanning electron microscopy (Fig. 2). Sections showed scanty hyphae predominantly on the outer surfaces of the paper, lying alongside the cellulose fibres without penetrating them (Fig. 3). The

Fig. 2 Scanning electron micrograph of a foxed lesion. Only three hyphae are present (one marked by arrow), most fibrils being cellulose. Sputter-coated with gold and examined in a Cambridge Stereoscan S600 at 25 kV. Fixation and critical-point drying were omitted to avoid washing material from the surface of the paper. Scale bar 20 μm .



fibres also appeared normal when examined *in situ* by phase contrast microscopy or dark-field microscopy, or after samples had been teased apart in Herzberg's zinc chloride–iodide stain¹⁹. Those findings were consistent with all the lesions being intact, although they were more easily wetted than normal^{10,11}. This was obvious when samples were floated on 0.25% (w/v) aqueous Coomassie blue which penetrated the lesions almost instantaneously. When the whole of the paper had become stained and was then differentiated by washing in water²⁰, some lesions showed a regular target-like structure (Fig. 4).

Viewed under an ultraviolet lamp (Hanovia 16744/1) through ultraviolet-absorbing goggles, foxed areas stood out as white or shining against a dark bluish-grey background²¹, although they might show almost no browning by daylight. After any browning had been removed by oxidising bleaches (ClO_2 solution²² or 5% (w/v) KMnO_4 followed by 2% (w/v) oxalic acid²³), the lesions still differed in colour under ultraviolet light and usually had fluorescent patches. When unstained paper was examined by fluorescence microscopy, the brightness of the cellulose clearly increased within the lesions but, apart from a suggestion of fluorescence at the margins of cellulose fibres, its exact site could not be determined.

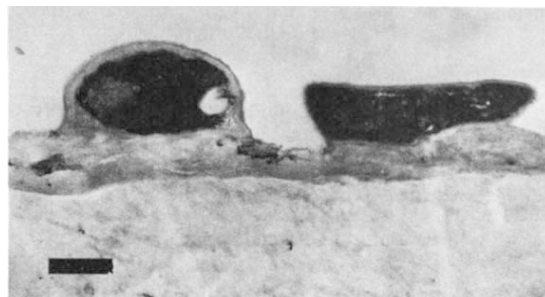


Fig. 3 Transmission electron micrograph of fungal hyphae on the surface of a cellulose fibre. They are in close contact with the outer layer of the fibre, but there are no structural changes associated with their presence. Fixed with buffered glutaraldehyde and osmium tetroxide, embedded in Spurr's resin²⁵, sectioned with a diamond knife on a Reichert OMU3 ultramicrotome, stained with uranyl acetate and lead citrate, and examined in an AEI 801A electron microscope at 60 kV. Scale bar, 0.5 μm .

Lesions also differed chemically from their surround. In addition to being more acid^{2,5}, they were invariably strongly ninhydrin-positive when sprayed with 0.5% (w/v) ninhydrin in acetone (butan-1-ol or ethanol were far less satisfactory, possibly because they penetrated the paper less well). Each ninhydrin-positive area corresponded to the area of fluorescence, not to the area of browning which was often far smaller. Here again, the lesions often had a differentiated structure, with the edge staining more strongly than the centre.

These findings suggest that when paper becomes sufficiently damp, fungal spores germinate and mycelial development begins, particularly at sites relatively rich in nutrients, such as the printed area of the page which receives the oily vehicle of the ink (ref. 2, page 415) or the fore-edge of the book which picks up nutrients from the reader's hands. However, the lesions evidently progress extremely slowly, either because fungal growth is intermittent (for example, because the temperature falls or because the book is opened occasionally in a warm room and dries out) or because nutrient is scarce and is exhausted before much growth has occurred. As a result, hyphae are generally scarce in the lesions and may be dead or absent from many parts of them, and the paper therefore never looks mouldy. Judging from the present examples, the fungi grow on the size, not the cellulose, of the paper: hence, the lesions are easily wetted^{10,11} but are otherwise structurally intact. Langwell¹⁰ emphasised that foxing led to perforation

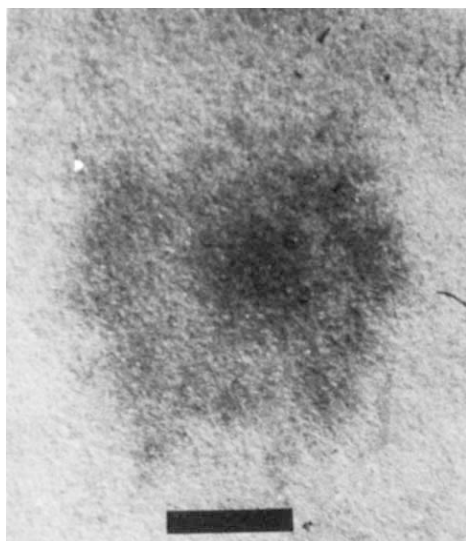


Fig. 4 Foxed lesion, showing target structure seen after staining with aqueous Coomassie blue and differentiation in water. The bar represents 5 mm.

but what seems more striking, considering how rapidly cellulolytic organisms can destroy paper¹² and that foxed papers are usually several decades old, is how rarely perforation is seen. When it does occur, it might as well result from the acid produced by fungal metabolism as from enzymatic attack. The positive ninhydrin reaction reflects either digestion of the size (filter paper sized with 5% (w/v) gelatin is ninhydrin-negative) or the presence of fungal products which are presumably also responsible for the fluorescence, as in ringworm (though reaction of amino compounds with cellulose is a possibility²⁴). Browning can be triggered by daylight⁷ and, considering the enormous number of coloured microbial metabolites, there seems no reason to postulate that iron is involved, particularly as the lesions contain no more iron than the surrounding paper^{5,8}. Prevention of fungal attack is generally agreed to depend primarily on maintaining a sufficiently low relative humidity (<70%) during book production⁶ no less than in libraries^{10,11}. However, one predisposing cause seems not to be mentioned: the sheets of tissue paper often used to prevent offsetting of engravings onto their facing pages. Tissue paper is unsized and will always tend to be damp and to absorb size from the adjoining paper. Not surprisingly, one repeatedly sees books where virtually all the foxing has occurred in those sheets originally intended for protection.

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Gaseous emissions of mercury from an aquatic vascular plant

RECENT studies have demonstrated the emission of mercury to the atmosphere from a variety of sources including contaminated industrial waste beds^{1–4}. Investigations have also shown that vascular plants can take up both inorganic and organic mercury compounds through their root systems and transport them to the leaves^{5,6}. Separate measurements of mercury emanations from the excised leaves of three vascular plant species have shown that there is a release of volatile mercury at room temperatures⁷. Thus, it would seem that vascular plants have the potential to take up mercury from a substrate, to transport it, and to emit it to the surrounding atmosphere through their leaves. However, no direct measurements of such emissions from living intact plants have been reported. We report here our examination of the common reed, *Phragmites communis*, growing along the shoreline of a mercury-contaminated lake and our finding that these plants, growing in their natural environment, actively release mercury to the atmosphere.

Two sampling stations were located on Onondaga Lake in Syracuse, New York, at two points of tributary inflows which formerly contributed large discharges of mercury to the lake from a local manufacturing plant. A control station was located on a stream near White Lake, an uncontaminated natural area south of Syracuse. At each of these stations, mercury emissions from individual *P. communis* plants growing amidst extensive reed belts were measured and compared.

The sampling apparatus consisted of a two-piece 12 × 110 cm cylindrical glass enclosure equipped with standard taper joints. The bottom piece was sealed to the plant stems with plasticine clay. Lowering the top piece over this created an air-tight seal. Air was continuously drawn through the enclosure at the rate of about 3 l min⁻¹ using a vacuum pump. Incoming air was cleaned of its mercury content by a gold-plated glass bead trap placed at the inlet hole. An assessment of these gold bead traps for quantitative removal of elemental mercury and a wide range of inorganic and organic mercury compounds from an air stream has been previously described⁸. Any mercury emitted from the leaves of the plants enclosed within the glass chamber was entrained in the mercury-free air stream and collected on the gold bead sampling trap. The sampling traps were subsequently analysed for total mercury by the plasma emission spectroscopy method of Braman and Johnson⁸. Detection limits of this technique are of the order of 20 × 10⁻¹² g of Hg. Before each measurement period, the large glass plant-enclosures were degassed of mercury for 15 h in a 550 °C annealing oven.

The emission of mercury was measured from living plants at the sampling sites for 2–4-h periods. Eight emission samples were collected from plants at different growth stages at each of the sampling stations during the summer of 1977. Before and after each measurement, a blank was run by drawing air through the chamber (without the plant) for a time corresponding to the sampling period. All the results were corrected for the measured low blank values. After each sampling

period, the dry biomass (105 °C) and the leaf area (BP-8 video image analyser) of the sampled portion of the plants were determined.

During the investigation eight sediment samples were collected from each study site close to the plants being measured. Analyses for total mercury in these sediments were carried out by a high temperature (>800 °C) combustion, plasma emission spectroscopy technique⁹. The top 6 cm of each sediment core were removed and homogenised before analysis.

To determine if a diurnal variation of mercury release existed, two different plants with a measurable Hg emission were monitored continuously for 26 h, changing the sampling traps every 2–4 h during daylight. A 10-h sampling period during darkness was also included in the study.

As the release of mercury was apparently linked to transpiration and/or photosynthesis (see discussion below), the mercury emission values were normalised to the surface area of the leaves enclosed within the chamber. Mercury release was also a function of the ambient air temperature. Because the air temperature on different sampling days ranged over 18–31 °C, the emission values were also corrected by a factor corresponding to a ratio of the vapour pressure of Hg at the temperature on the given sampling day. Normalising the results to leaf area and the vapour pressure correction factor provided the smallest coefficient of variation at each site.

The results (Table 1) clearly show a significant release of mercury by the plants growing on the shoreline of Onondaga

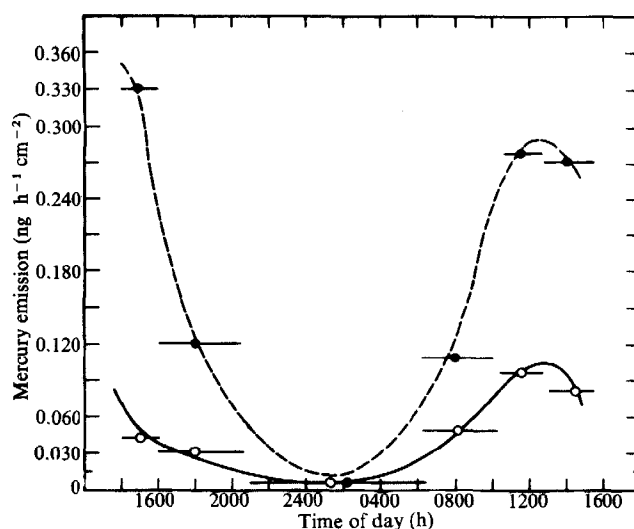


Fig. 1 Diurnal variation of mercury emission from *P. communis*. The open and closed circles denote two separate experiments at Lake Onondaga.

Table 1 Mercury emission values and mercury concentration in the surface sediments

Station	Mercury emission from <i>P. communis</i> (ng h ⁻¹)	Mercury emission corrected by vapour pressure ratio and normalised to leaf area (ng h ⁻¹ cm ⁻²)	Mercury concentration in surface sediments (μg g ⁻¹)
I	1.3	0.015	3.4
Onondaga	ND*	ND	2.8
Lake at	14.1	0.073	2.5
Nine Mile	27.2	0.076	4.5
Creek	11.3	0.038	3.2
	6.0	0.064	2.4
	7.3	0.047	3.2
	ND	ND	3.2
Mean	8.4 ± 8.6	0.035 ± 0.027	3.15 ± 0.7
II	17.6	0.271	8.7
Onondaga	13.4	0.337	10.1
Lake at	20.1	0.215	17.3
East Flume	16.8	0.093	4.3
	9.9	0.059	14.5
	5.9	0.051	13.8
	6.5	0.041	6.1
	24.5	0.330	8.3
Mean	14.3 ± 6.2	0.175 ± 0.10	10.4 ± 4.2
III	6.0	0.029	0.03
White Lake	ND	ND	ND
	5.4	0.012	ND
	3.6	0.018	0.13
	ND	ND	0.05
	1.6	0.009	0.03
	ND	ND	0.17
	ND	ND	0.20
Mean	2.9 ± 2.5	0.009 ± 0.011	0.08 ± 0.07

* ND, nothing detectable.

Lake as compared with the low emissions at the control site, and suggest a positive relationship between the emission of mercury by the plants and the concentration of mercury in the surface sediments. Although we were not concerned here with the form of mercury released, four auxiliary analyses by cryogenic trapping with subsequent gas chromatography/plasma emission spectroscopy indicated only the presence of elemental mercury in the samples (D. Gay, personal communication).

The pattern of mercury release is distinctly diurnal (Fig. 1). The absence of mercury emission during dark hours, when the stomata of the plant are closed, as well as the occurrence of maximum mercury release during the period of maximum light conditions and maximum transpiration rate of *P. communis*¹⁰, suggest a link of mercury emission with transpiration and/or photosynthesis.

Hence, vascular plants may play an important part in the removal of mercury from soils and sediments by injecting it directly into the atmosphere. Further, our results show that the extent of these emissions is related to the mercury concentration in the substrate and the diurnal pattern of the plants' physiological processes.

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Effects of thermal effluent on body condition of largemouth bass

PAR POND, an 1,100-hectare reservoir in South Carolina, has received heated effluent from a nuclear production reactor since 1957. A notable feature of the reservoir¹ is the abundance and abnormally high catchability of largemouth bass (*Micropterus salmoides*²). Most reported responses of bass to the thermal situation in Par Pond are interpreted as inconsequential³⁻⁷; increases in the densities of certain intestinal parasites⁸ and the incidence of red-sore disease⁹ in thermal areas are considered detrimental. Fishery scientists use body condition¹⁰ as an index for estimating a fish's well-being or fitness¹¹ as well as the relative suitability of the habitat¹². Body condition (K) of an individual is established by the relationship $K = 10^3 W \times L^{-3}$, where W = body weight (g) and L = standard length (mm). However, caution must be exercised to ensure that ontogenetic changes in body shape, seasonal and sex differences, and small sample sizes do not preclude the effective use of the index for comparative purposes among individuals¹⁰. This report takes these reservations into account and compares body condition of largemouth bass collected from heated and unheated waters in Par Pond. The unique feature of this study is that data from 10 years are available on a large sample ($n > 10,000$) of largemouth bass, an important species of warmwater sport fish. Lower body condition is associated with moderate thermal conditions comparable to many industrial settings and is correlated with lower levels of stored fat and with the incidence of red-sore disease.

Bass were collected primarily by angling or electrofishing, and the following data were obtained: capture date, location within the reservoir, standard and or total length, and body weight. Body condition (K) was determined using the standard

length of each fish. The regression of standard and total length indicated a highly predictable relationship ($r = 0.999$; $n = 3,000$) between these two measurements. So, if only total length had been taken on a particular fish, standard length was estimated for calculating K values. To eliminate the juvenile component from the comparisons and to preclude problems emanating from ontogenetic changes in shape and symmetry¹⁰, only individuals ≥ 20.0 cm standard length are included in this study.

Par Pond receives moderate thermal loading equivalent to power reactor levels creating surface temperatures with ΔT values of up to 10 °C in the heated areas¹³. Mean maximum surface temperatures during reactor operations in summer months were 35 °C in thermally-altered areas and 31 °C in unaffected locations; in winter mean temperatures were 20 °C in heated areas and 16 °C at natural sites¹⁴. Complete descriptions of the physical, chemical and biological characteristics of Par Pond are given in other reports^{1,15}. Bass taken within 200 m of the effluent (the area of maximum elevated temperatures) were classed as being from the heated area; those from other locations in Par Pond were grouped for analysis as being from unheated habitats. The unheated sampling areas were 2–7 km from the heated area. Substantial evidence exists to indicate that some Par Pond bass occasionally move long distances in the reservoir¹⁶, although movement between distant regions is minimal^{17,18}. Hence, a particular section of lake has a relatively discrete subpopulation that maintains its integrity relative to other sections.

There is a strong correlation between body condition of largemouth bass and the location of capture relative to the heated effluent (Table 1). Adult fish from unheated areas exhibited significantly higher mean condition factors than those from the heated area. Condition factors were usually higher in both heated and unheated areas during winter than during any other season. Lowest condition factors occurred in unheated areas in spring or autumn of most years, whereas summer was always the period of poorest body condition for bass in the

Table 1 Mean body condition coefficients (K) of largemouth bass (*Micropterus salmoides*) from a South Carolina reservoir receiving heated effluent

Season*	Winter		Spring		Summer		Autumn	
	H	U	H	U	H	U	H	U
1967	—	—	—	—	1.99 (17)	2.17 (27)	—	—
1968	—	—	—	—	—	—	—	—
1969	2.32 (187)	2.41 (174)	2.12 (281)	2.27 (195)	1.97 (44)	2.31 (419)	2.00 (335)	2.21 (59)
1970	2.28 (62)	2.35 (211)	2.12 (148)	2.17 (201)	2.12 (33)	2.32 (296)	2.19 (18)	2.14 (13)
1971	2.22 (273)	2.14 (61)	—	—	1.85 (87)	1.94 (79)	2.01 (18)	—
1972	2.54 (23)	2.46 (19)	—	—	2.10 (52)	2.32 (274)	2.09 (46)	2.20 (276)
1973	2.30 (65)	2.38 (331)	2.08 (55)	2.14 (200)	1.91 (210)	2.19 (181)	1.97 (43)	2.32 (131)
1974	—	—	—	—	—	—	2.14 (115)	2.28 (57)
1975	2.26 (366)	2.50 (228)	2.25 (457)	2.38 (447)	1.87 (432)	2.12 (562)	2.41 (153)	2.41 (112)
1976	2.37 (164)	2.44 (98)	2.30 (200)	2.33 (272)	2.02 (201)	2.20 (207)	2.05 (246)	2.16 (160)
Total	2.29 (1140)	2.39 (1122)	2.20 (1141)	2.28 (1315)	1.93 (1059)	2.25 (2018)	2.10 (974)	2.25 (808)

Sample sizes are indicated in parentheses. H, fish collected from waters heated by thermal effluent; U, fish collected in unheated, normal temperature portions of lake, 2–7 km from H. Standard errors for season and location of individual years ranged from 0.01 to 0.05. Body conditions of all bass from thermal areas in a particular season are significantly lower ($P < 0.05$) than those during the same season from natural areas.

* Winter = December, January, February; summer = June, July, August; spring = March, April, May; autumn = September, October, November.

Table 2 Standard lengths ($\bar{x}$ cm) of bass from Par Pond reservoir in different seasons

Season	Males		Females	
	H	U	H	U
Winter	32.1 $\pm$ 0.19 (270)	33.4 $\pm$ 0.13 (356)	35.6 $\pm$ 0.19 (354)	35.8 $\pm$ 0.17 (425)
Spring	32.3 $\pm$ 0.24 (112)	32.8 $\pm$ 0.16 (271)	35.7 $\pm$ 0.16 (365)	35.4 $\pm$ 0.18 (355)
Summer	32.3 $\pm$ 0.15 (199)	32.8 $\pm$ 0.11 (524)	35.4 $\pm$ 0.17 (225)	35.6 $\pm$ 0.13 (597)
Autumn	32.2 $\pm$ 0.36 (82)	32.4 $\pm$ 0.20 (257)	36.6 $\pm$ 0.38 (102)	34.6 $\pm$ 0.33 (203)

The data show that adult female bass from this reservoir are significantly larger than adult males; however, no length differences are apparent between different seasons or thermally different locations. Comparisons are based on samples taken between 1969 and 1973 in which sex was determined by dissection. H, heated habitats; U, natural temperature habitats. Values are means $\pm$ s.e.m. with the number of determinations shown in parentheses.

heated area. Females ($\bar{x}$ = 35.5; s.e. = 0.067, n = 2,626) from all locations and during all seasons were significantly longer (standard length in mm) than males ($\bar{x}$ = 32.6; s.e. = 0.059; n = 2,071). However, no consistent difference in condition factors was observed between the sexes. Average fish length within each sex was relatively constant between seasons and locations; (Table 2). Thus seasonal and locational differences in condition factors are attributable to differences in individual weights in otherwise similarly-sized fish. No consistent trend in body condition was apparent from one year to the next over the 10-yr sampling period (Table 1).

The amount of body fat that can be removed by dissection is minimal in individuals having low body conditions (Fig. 1). Although the relationship is not readily definable to a linear or curvilinear model, bass with body conditions less than 1.8 have no intracoelomic fat reserves. Thus, fish with low body condition are low in stored energy reserves.

The simplest explanation for the differences in body condition and body fat levels among adult Par Pond bass is as follows. (1) Although some transient fish are attracted to the effluent, most bass inhabiting the heated area of the reservoir are permanent residents¹⁶⁻¹⁸. (2) Although bass in the heated area may frequent cooler, stratified waters, a sufficient proportion of time is spent in the heated layer to elevate body temperatures³. (3) An elevation in temperature of largemouth bass results in an increase in metabolism¹⁹. (4) Fat reserves are called upon when food intake is insufficient to meet energy requirements of some vertebrates, including fish²⁰. (5) Metabolic demands would be elevated during the warmer months when body temperatures are highest (D. H. B., unpub-

lished). (6) Frequent observations indicate that population levels of forage fish, primarily *Lepomis* spp., the mainstay in the diet of adult bass from Par Pond⁴, are dramatically reduced during these warmer months. Also, associated with the advent of warmer weather is the greater expenditure of energy and loss of weight as a consequence of spawning, thus, the combination of increased metabolism, reduced food intake, reproductive activity and depletion of intracoelomic fat reserves results in a loss of weight and reduced body condition. These findings draw attention to subtle biological effects of thermal pollution that may go unnoticed without critical, systematic examination on a long-term basis.

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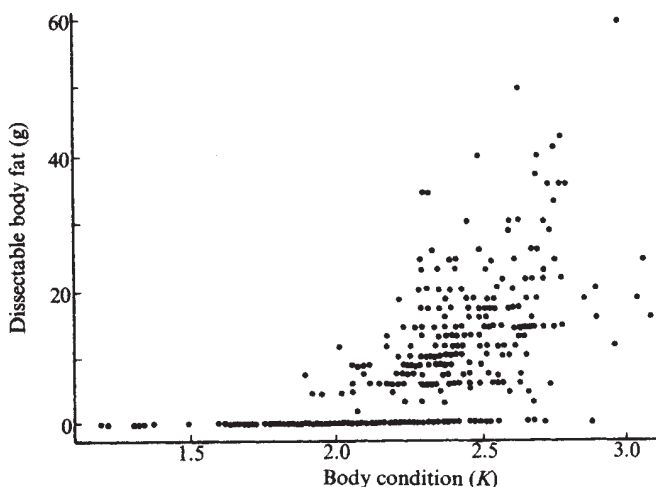
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Fig. 1 Relationship between body condition and intracoelomic fat reserves of largemouth bass (*Micropterus salmoides*; n = 275) taken during the summer and autumn of 1973 from a thermally influenced reservoir in South Carolina.



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Normal and experimental variations in proportions of skeleton of chick embryo wing

THE precise control of symmetry between left and right paired skeletal elements of the developing chick embryo can be exploited in a sensitive test of the effect of unilateral experimental perturbation¹⁻⁴. I report here a modified technique which can be used to detect localised defects without reference to the contralateral side. This can give a clear indication of the teratogenic effect of irradiation and various chemical treatments at doses smaller than those usually necessary to demonstrate such effects. Such experiments are of necessity bilateral in their effect unless laborious interembryonic grafts are performed (C. Tickle, personal communication).

Table 1 Proportions of skeletal elements

	Humerus	Ulna	Digit III
Mean proportion (% total length)	35.73	29.94	34.32
Standard deviation	0.74	0.64	0.90
Mean length for sample	4,219 μm	3,535 μm	4,052 μm
95% Limits (ipsilateral ratio)	175 μm	151 μm	228 μm
95% Limits left:right	109 μm	95 μm	164 μm

The basis of the test is the observation that the parts of the skeleton of a single limb occupy a relatively constant proportion of the total length during development¹⁰, a fact well documented in physical anthropology and forensic work^{5,6}. Limb abnormalities caused by teratogens applied for a short time are level specific^{7,8}, and the consequent reduction changes the proportion of the limb occupied by that level.

The normal control consisted of 84 White Leghorn embryos fixed between days 9 and 11 of incubation. They were then stained with alcian green 2GX and cleared in methyl salicylate and the lengths of the humerus and the ulna and the combined

Fig. 1 Response at day 10 of embryos from stages 12–16 to various doses of X-ray irradiation. Mortality: percentage of embryos dead; gross defect: percentage of embryos showing a gross skeletal abnormality; malproportion: percentage of embryos showing a significant (95% level) deviation from normal proportions. Bars represent one standard error.

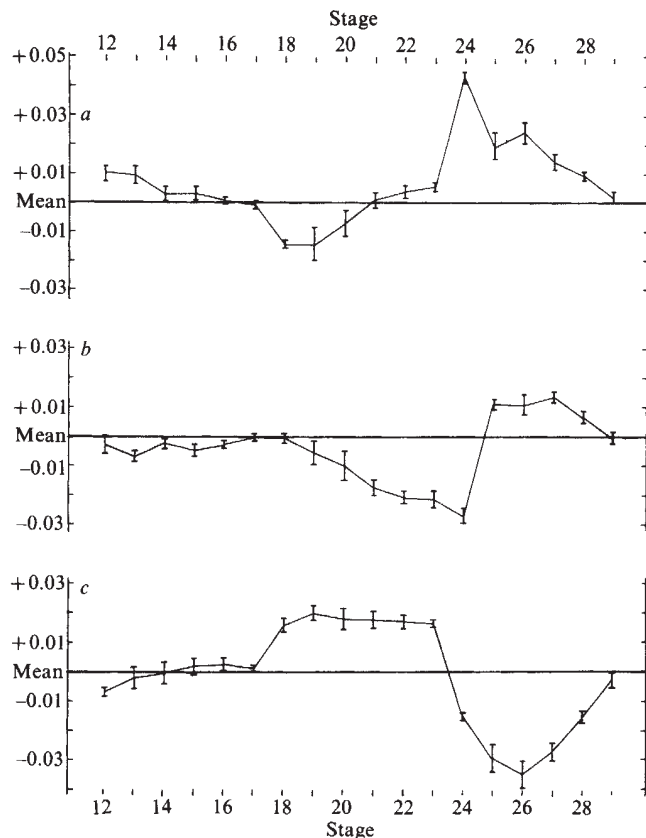
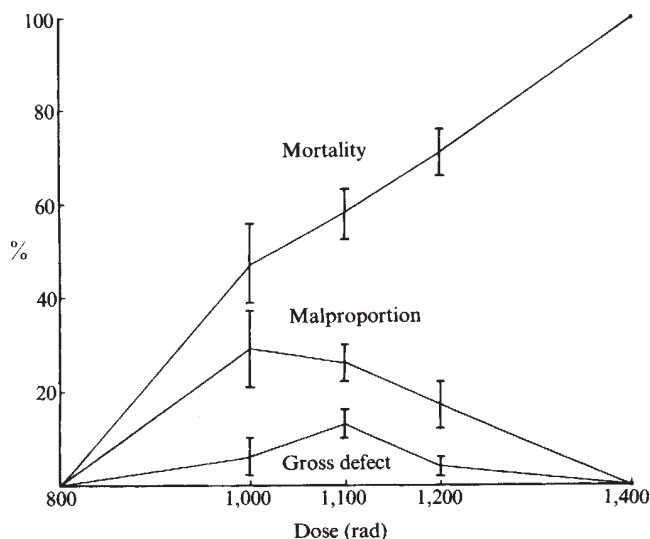


Fig. 2 The heavy centre line represents the normalised mean ratio between the skeletal element and total wing length in a normal population. Above the line is longer than normal, below the line is shorter than normal. The standard error for the population mean is less than 10^{-4} and is not shown. The bars show the mean and standard error for the ratio between skeletal element and total wing length for all the embryos at each stage which did not show a gross defect. a, Humerus; b, ulna; c, digit III.

length of the metacarpal and phalanges of digit III were measured as described previously¹. The ratios of the lengths of each measured element to the total limb length are shown in Table 1 together with the standard deviation and the mean length of the sample for each element. Ignoring the wrist which does not grow during this period^{9,10}, it is clear that this strain of embryos maintains relatively constant proportions. A limb 12,000 μm long will have a humerus of $4,219 \pm 175 \mu\text{m}$, an ulna of $3,535 \pm 151 \mu\text{m}$ and a digit III of $4,052 \pm 228 \mu\text{m}$ in 95% of the population.

We can compare this with the precision achieved when comparing left and right elements in the same embryo¹, the equivalent 5% confidence intervals being: humerus $\pm 109 \mu\text{m}$, ulna $\pm 95 \mu\text{m}$, and digit III $\pm 164 \mu\text{m}$. Clearly both within embryo and between embryo variations represent a remarkable degree of precision, equivalent (as argued previously¹) to an error in initial specification of 1 cell in 20 (5%).

I now present one experimental example to illustrate this method. Developing embryos were irradiated using a Marconi high voltage X-ray machine at 230 keV with a dose rate of 1,000 rad min^{-1} . The dose range was 800–2,000 rad and the stage of embryos varied from 14 to 28 (ref. 11). Embryos were allowed to develop until the 10th day of incubation when survivors were fixed, stained and cleared, and the ratios of the lengths of each element to the total limb length were calculated.

X-ray irradiation produces defects which are stage dependent⁷. One of the problems of analysis is that the optimum teratogenic dose¹² (the dose producing the maximum number

of living embryos with a detectable abnormality) is lethal for more than half the sample (Fig. 1). Furthermore, the teratogenic dose range¹² (any dose that can cause a detectable abnormality) is narrow, and the teratogenic potency¹² (the percentage of malformed embryos produced by the optimum teratogenic dose) is a small proportion of the survivors. If the estimate of these parameters includes those limbs in which there is a significant malproportion, then the situation is much improved. In my example (Fig. 1) I include all limbs in which one element varied by more than two standard deviations from its normal proportion, and as a result the estimation of the optimum teratogenic dose changes from 1,100 rad to 1,000 rad, and the teratogenic potency increases from $11 \pm 3\%$ to $29 \pm 8\%$.

This method can also give a more accurate assessment of the effect of treatment. Figure 2 shows the effect of X-ray irradiation on the limb for stages 14 to 29. The heavy centre line represents the mean ratio between the humerus (Fig. 2a), the ulna (Fig. 2b), and digit III (Fig. 2c) and the total limb length for the control series. The standard error for the mean is less than 10^{-4} and is not shown. Each experimental point represents the appropriate mean ratio at a particular stage together with the standard error of the mean. The data are drawn from all limbs irradiated within the teratogenic range in which gross skeletal abnormalities were absent. The figure shows clear level-specific defects at each stage. For example, at stage 18 the humerus is comparatively short compared with both ulna and digit III.

I have used the system to test bromodeoxyuridine, cytochalasin and oligomycin. In each case it was possible to show that the method was more sensitive in detecting a teratogenic effect. The detailed results will be reported elsewhere.

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Anterior and posterior compartments in the head of *Drosophila*

A COMPARTMENT¹ is a precisely demarcated region of an adult insect which is made by a group of related cells. This group (a polyclone²) consists of all the descendants of several founder cells which were set aside at an earlier stage. The developmental pathway followed by a growing polyclone depends on the local state of a small number of controlling 'selector' genes³. One example is the *engrailed* gene which is thought to determine the 'posterior' rather than the 'anterior' pathway of development and is used in several different segments of the developing fly^{4–6}. The thorax of *Drosophila* is

constructed on these simple principles; each of the three segments consists of two polyclones which generate the anterior and posterior compartments^{1,7}. The proboscis is similarly subdivided⁸ but the abdominal segments are probably not⁹. The segmental status of the head is controversial^{10–12}. Here we summarise three lines of evidence which show that the main part of the 'head'—the cuticular parts formed by the eye-antennal disc¹³—is constructed on the same principles as a thoracic segment.

To study cell lineage in the head (eye, head capsule, maxillary palp and antenna)¹³ we have made fast-growing clones, marked with cuticular and bristle mutants, at defined stages of embryonic and larval development^{14–17}. When made in larvae younger than 48 h after egg laying (48 h AEL), these clones sometimes fill the entire head on one side and respect no particular boundaries. After 48 h AEL they respect a local line dividing the eye into dorsal and ventral halves^{14,18–20}, although they overlap elsewhere. Clones made at 72 h AEL, or later, fall into only two classes (Table 1); some mark territory in an A region which includes much of the head capsule, all the eye and a defined region of the antenna, whereas others mark the remainder, or P region (Fig. 1). The two sets of clones meet each other along one continuous complete boundary. This shows that by 72 h AEL there are two polyclones in the eye-antennal disc, which will make the A and P compartments, respectively.

In the *engrailed* mutation (*en*¹/*en*¹) the posterior compartments of all three thoracic segments are defective, and sometimes bear structures of anterior character. These anterior patterns can be arranged in mirror symmetry with the normal

Fig. 1 Diagram of frontal aspect of head to indicate P (shaded) and A (unshaded) regions. The fly's left antenna (on the right of the diagram) has been folded back and rotated through 180° to show the underlying cuticle and its undersurface (decorated with posterior *Zahnborsten*). The boundary between A and P regions skirts the raised prefrons (pr) and the vibrissal area (v) and was approximately mapped from *mwh Minute*⁺ clones generated in a *Minute* background. The limits of the A/P boundary in the palps have not been precisely defined. Certain bristles, which are near the boundary, can be marked by both A and P clones (arrows).

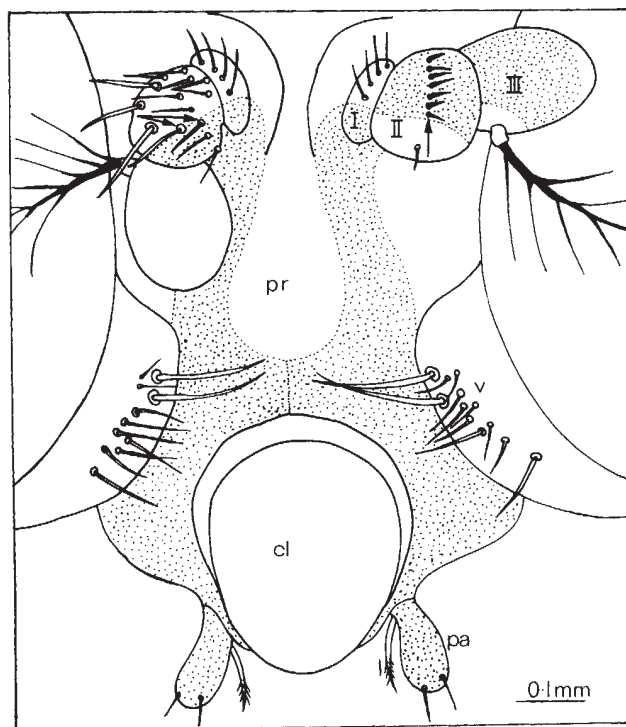


Table 1 Fast-growing clones, marked with cuticular and bristle mutants, made at defined stages of development

Age (h AEL)*	n	A	P	A + P	No. of clones†
0-28	2,876	54	20	19	0.4
48 ± 4	806	29	17	6	0.6
72 ± 4	962	54	22	2	1.2

Flies were $M(1)o^{Sp}/yw f^{36a}$ and irradiated with 500R by standard methods²⁵.

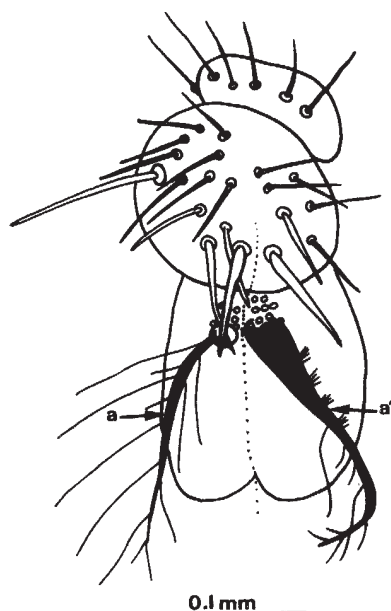
* The larvae are *Minute*, so timing does not correspond to wild-type flies^{17,26}.

† Number of clones expected in both A and P regions simultaneously (when the result of two independent events).

anterior pattern (refs 4-6, and unpublished). Anterior compartments are untouched by the mutation. A new allele of engrailed (en^2) has been isolated by T. Kornberg; flies mutant for en^1/en^2 show strong effects on the thoracic segments and the antenna. The antennal pattern of bristles is considerably altered, extra A-like bristles form in the P region, and frequently an adventitious arista is formed on the P side of the second segment. This gives the antenna a partial mirror symmetry, the mirror plane coinciding with the A/P border (Fig. 2). Apparently, with respect to function of the engrailed gene, the P region of the antenna is posterior and the A, anterior.

In mutants of *spineless-aristapedia* the distal parts of the antenna are replaced by tarsus, whereas the rest of the head is unaffected²¹. This homeotic transformation is clearly expressed in flies of the genotype $ss^a/In(3LR)P88, ss$ (ref. 21) which we have used in an analysis of cell lineage. Large clones were produced in these flies by standard techniques¹⁷. Before 72 h AEL clones can cross from anterior to posterior tarsus, or even fill the entire structure. After that, clones are confined to the A region, which includes anterior tarsus, or to the P region including posterior tarsus. The border respected by clones in the homeotic tarsus is in exactly the same place as the compartment border in the normal tarsus (unpublished).

Fig. 2 Exterior aspect of an antenna of a fly showing a strong engrailed phenotype (en^1/en^2). Compare with Fig. 1 and note the extra bristles, the apparent mirror plane (dotted line), the bilobed third segment (III) and the adventitious arista a'.



Our results show homology between the A and P regions of the head and the anterior and posterior thoracic compartments. We suggest the mutant *aristapedia* reveals the homology by transforming part of A into anterior tarsus and part of P into posterior tarsus. In both wing and antenna the *engrailed* mutation produces a partial duplication of the anterior pattern. This observation illustrates the genetic basis of the homology. We therefore suggest that the A and P regions are true compartments.

The mutant *Nasobemia*, which sometimes produces a perfect mesothoracic leg in place of the antenna²², presumably transforms the entire A region in the antenna into an anterior leg compartment, and the entire P region into a posterior compartment. Our results show that all the eye and most of the head is related by cell lineage to the A region of the antenna, and other mutants (for example, *Ophthalmoptera* (ref. 23, and W. K. Baker, unpublished) confirm that the A region in the head is homologous to the anterior mesothorax.

Not all the properties of the anterior and posterior compartments of the head are the same as those of the thorax and proboscis; clones can cross from A to P regions of the head during larval life, whereas in the thorax and proboscis, anterior and posterior polyclones are defined at about the blastoderm stage^{7,24,25}. This would mean that, under our hypothesis^{5,6}, the *engrailed* gene is activated later in the head than elsewhere. In spite of this difference we draw two conclusions: that the head capsule, the eye, the antenna and the so-called 'maxillary' palp belong to one segment; and that the head and thoracic segments are constructed similarly.

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Stimulation of uridine uptake in 3T3 cells is associated with increased ATP affinity of uridine-phosphorylating system

CELLS maintained in culture may be brought into a quiescent state by transfer to suboptimal medium and subsequently activated to proliferate by addition of complete medium¹. An early event during activation with serum is the stimulation of uridine uptake². Recent work has shown that it is not uridine transport but the subsequent trapping of uridine within the cell, that is enhanced^{3,4}. We report here a study of the trapping of uridine by 3T3 fibroblasts *in situ* in which we have varied separately both the uridine and the ATP levels within the cells. Stimulation of uridine uptake as a result of serum or insulin activation was found to be associated with a dramatic increase in the half-saturation concentration of the uridine trapping system for the substrate ATP, with relatively little change in the maximum velocity of uptake or in the affinity for uridine.

The time course of uridine uptake by quiescent and serum-activated 3T3 fibroblasts at several uridine concentrations is shown in Fig. 1a. These curves are essentially linear over the period studied and the slopes of such lines give the velocity of trapping of uridine, since the c.p.m. per dish observed at all points after 10 min exceeds 200 c.p.m. per dish, estimated for uridine at transport equilibrium. That the trapping of uridine into acid-soluble pools represents phosphorylation has been shown by thin layer chromatography of trichloroacetic acid-soluble extracts⁵. We are therefore justified in taking the slopes of such curves as a measure of the rate of *in situ* phosphorylation.

Uridine kinase, the rate limiting enzyme for uridine phosphorylation in 3T3 cells⁵, utilises as substrates in this bisubstrate reaction, ATP and uridine⁶. Thus, theoretically, stimulation of uridine phosphorylation could be seen as an enhanced activity of the system in response either to uridine or to ATP, or to both of these substrates. To ascertain which of these possibilities is in fact involved we undertook separate kinetic analyses of the interaction of each substrate with the uridine phosphorylating system.

Figure 1b shows a Lineweaver-Burk plot of the trapping of uridine by quiescent and serum-activated 3T3 fibroblasts as a function of the extracellular uridine concentration. Linear regression analysis of these plots gave values (mean $\pm$ s.e.) of $K_m = 7.3 \pm 1.3 \mu\text{M}$ and a $V_{\max} = 1.56 \pm 0.24$ pmol per min per dish for quiescent cells and a $K_m = 9.2 \pm 1.9 \mu\text{M}$ and a $V_{\max} = 3.62 \pm 0.67$ pmol per min per dish for serum-activated cells. Thus serum-activation produces more than a twofold increase in $V_{\max}$ without a significant change in K_m , when uridine is the varying substrate. (We recognise that the pertinent concentration of uridine is its intracellular value rather than extracellular levels used in Fig. 1b. Unpublished calculations, using the measured values of the transport kinetic parameters for uridine, show that for the data in Fig. 1b the intracellular concentration is only 20% less than the extracellular concentration at the K_m value⁷.)

To analyse the kinetics of uridine phosphorylation *in situ* as a function of ATP concentration, the intracellular level of ATP was set by incubation of quiescent and serum-activated 3T3 fibroblasts in different concentrations of 2-deoxyglucose⁸. This treatment resulted in a systematic reduction of ATP concentration, as demonstrated in Fig. 2, in the absence of uridine uptake. It is important to note that both the basal level of ATP and the level of intracellular ATP reached at any level of 2-deoxyglucose administered to the cell, were essentially identical in quiescent and serum-activated cells. The intracellular ATP concentration (mean $\pm$ s.e., 16 determinations) for cells

not treated with 2-deoxyglucose was 1.31 ± 0.07 mM for quiescent cells and 1.33 ± 0.09 mM for activated cells, consistent with reports comparing the nucleotide levels in growing and density-inhibited 3T3 cells⁹. Thus the increase in uridine phosphorylation that follows serum-activation cannot be attributed to an increase of intracellular ATP levels.

Using the 2-deoxyglucose technique to alter intracellular ATP concentration, a kinetic analysis of uridine phosphorylation was performed (Fig. 3). We measured, on the same dish, the intracellular ATP concentration and the amount of labelled uridine accumulated within the cells. The mean ATP concentration in quiescent and serum-activated cells is shown as a function of 2-deoxyglucose concentration in Fig. 3a. At each concentration of 2-deoxyglucose the ATP level did not change consistently during the time of uridine uptake (in 24 time courses the ATP level increased in 7, decreased in 8 and was not altered in 9 cases) and therefore, the points in Fig. 3a depict the mean intracellular ATP concentration during the

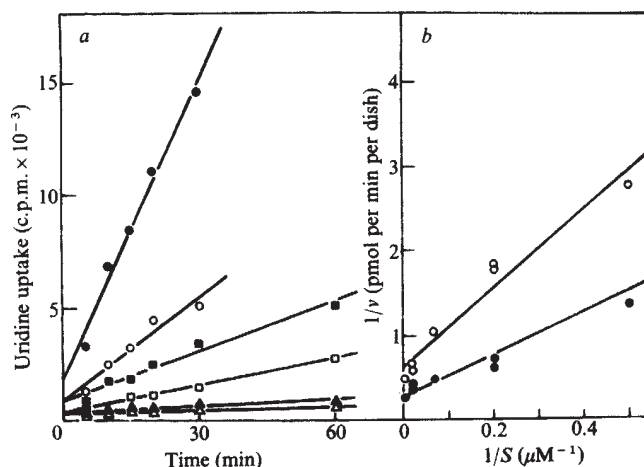


Fig. 1 *a*, Time course of uptake of H^3 -uridine by quiescent and serum-activated 3T3 fibroblasts *in vitro*. Fibroblasts, cultured in 25-cm plastic Petri dishes (Nunc) in Dulbecco's minimal essential medium (MEM) supplemented with 10% dialysed fetal calf serum (FCS) were incubated at 37°C in 5% CO_2 and humidified air. Cells were rendered quiescent by transfer to suboptimal medium consisting of 5% FCS in MEM for 10 days and in 0.25% FCS in MEM for 16 h. The experiment was initiated by replacing the suboptimal medium with approximately 1 ml of either 10% FCS in MEM (serum-activated cells) or 10% phosphate buffered saline (PBS) in MEM (quiescent cells) and the cells incubated at 37°C for 45–50 min. Activation or sham-activation was terminated by replacement with approximately 1 ml of either PBS (quiescent cells) or 10% FCS in PBS (activated cells) containing H^3 -uridine at a final concentration of $4 \mu\text{Ci ml}^{-1}$ and cold uridine at a concentration of $5 \mu\text{M}$ ($\circ$, $\bullet$), $50 \mu\text{M}$ ($\square$, $\blacksquare$) or $500 \mu\text{M}$ (Δ , $\blacktriangle$); open symbols are quiescent cells, solid symbols serum-activated cells. The cells were maintained at 20°C for 5 to 60 min, incubation terminated by washing with approximately 1 ml of ice cold PBS five times and the radioactivity extracted in 5% trichloroacetic acid at 4°C for 20 min. An additional 20% of radioactive counts appear as the acid-insoluble fraction (unpublished observation). The data are presented as uridine uptake in c.p.m. per dish plotted against time. In these conditions the equilibrium level for free uridine was calculated to be 200 c.p.m. per dish using the cell number per dish, the radioactive counts associated with a known volume of external medium and a cell volume of 5 pl, calculated from the equilibrium distribution level of 3-*O*-methyl glucose. *b*, Lineweaver-Burk plot of the uptake of H^3 -uridine by 3T3 fibroblasts in a quiescent state ($\circ$) and following serum activation ($\bullet$). The reciprocal of the uptake velocity (pmol per min per dish) was plotted against the reciprocal concentration of uridine (μM^{-1}). Uptake velocities were determined from linear regression analysis of time courses such as those illustrated in (*a*), taking into account the appropriate specific activity.

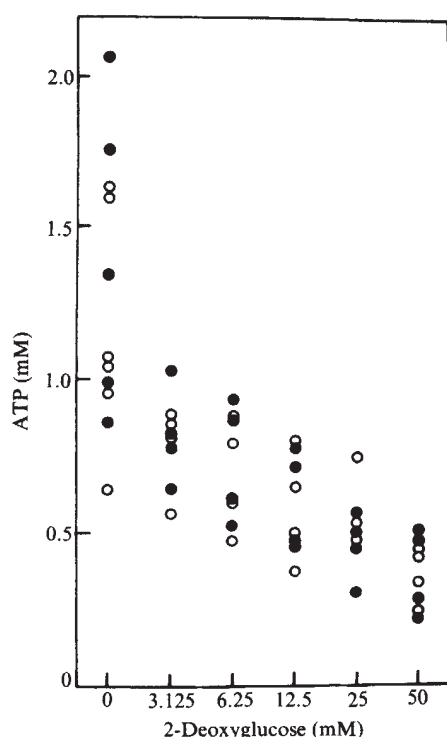


Fig. 2 Intracellular ATP concentration in quiescent (○), and serum-activated (●), 3T3 fibroblasts. Activated and quiescent cells were prepared as in Fig. 1a. The medium was replaced with approximately 1 ml of either 10% FCS in PBS (activated cells) or PBS (quiescent cells) containing various concentrations of 2-deoxyglucose, and incubated at 37°C for 15 or 30 min. (As no difference was noted in the results obtained at 15 and 30 min the data were pooled.) The incubation was terminated and the ATP extracted in 1 ml of boiling water, and the temperature maintained at approximately 80°C for 15 min. The ATP concentration was determined by the Luciferase assay¹⁰. The intracellular ATP concentration was calculated using the cell number (10^5 cells per dish) and the cell volume (5 pl). Each point depicts a single determination on a separate dish of 3T3 cells.

time in which uridine was being accumulated. The ATP levels in both quiescent and activated cells were lower than in cells which had not been exposed to uridine (compare Fig. 2 and 3a). For cells exposed to uridine but not to 2-deoxyglucose, the ATP levels were lower in activated than in quiescent cells. This is consistent with the greater degree of trapping of uridine in the activated cells and hence in their greater consumption of ATP. (In activated cells uridine uptake is approximately $40 \text{ pmol per } 10^6 \text{ cells (} \approx 5 \text{ } \mu\text{l) min}^{-1}$ or $8 \text{ } \mu\text{M min}^{-1}$ and would consume directly $24 \text{ } \mu\text{M ATP min}^{-1}$ or $360 \text{ } \mu\text{M}$ by 15 min, the incubation mid-point. Half of this uptake and corresponding ATP consumption will occur in quiescent cells. An additional 20% of such counts are incorporated into the acid-insoluble fraction which would include nucleic acids. Further ATP might be consumed in other phosphorylation reactions stimulated by uridine uptake). Thus uridine uptake leads to an ATP depletion over and above that achieved with 2-deoxyglucose.

Representative time courses of uridine uptake from the same experiment as that of Fig. 3a are shown in Fig. 3b. From such time plots we can generate a kinetic analysis of the rate of uridine phosphorylation as a function of the directly measured intracellular ATP concentration, at a uridine concentration 5 to 7 times higher than the measured K_m for uridine. The data for two separate experiments using serum activation are illustrated as Lineweaver-Burk plots in Fig. 4a. For quiescent cells, the K_m (mean $\pm$ s.e.) was $199 \pm 95 \text{ } \mu\text{M}$ and the V_{max} $11.4 \pm 4.6 \text{ pmol per } 10^6 \text{ cells per min}$ and for serum-activated cells, the K_m was $10 \pm 21 \text{ } \mu\text{M}$ and the V_{max} was $32.5 \pm 4.1 \text{ pmol per } 10^6 \text{ cells per}$

min. In the analogous experiments with insulin the measured ATP levels after deoxyglucose treatment approached zero, thereby rendering the Lineweaver-Burk analysis impractical.

To assess the data for serum and insulin-activation a computer analysis based on the Michaelis-Menten reaction was used (Fig. 4a, inset). The derived kinetic parameters and the range with 95% confidence error limits were: (1) In cells activated with serum or insulin the K_m was 0.097 mM (range 0.053 to 0.187) and the V_{max} was $43.1 \text{ pmol per } 10^6 \text{ cells per min}$ (range 34.1 to 52.3). (2) In quiescent cells, the K_m was 2.91 mM (range 2.89 to 4.22) and the V_{max} $100 \text{ pmol per } 10^6 \text{ cells per min}$ (range 74.5 to 126).

We prefer the computer analysis because it enables us to use all of the data and does not give undue weight to the points at low substrate concentration. However, using either analysis the K_m for quiescent cells is at least 20-fold greater than that of activated cells. This difference is so great that, within the range of ATP concentrations studied, uridine phosphorylation in serum-activated cells is almost always close to saturation, whereas in quiescent cells the system is well below saturation.

Several attempts to repeat these observations with enzyme preparations extracted from quiescent and serum-activated cells were undertaken. A kinetic analysis of a typical study using ATP as substrate revealed no difference in K_m for uridine phosphorylation between enzyme extracts from quiescent and activated cells and, in both cases, the K_m approximated to that obtained with quiescent cells *in situ* (Fig. 4b). There is clearly

Fig. 3 The simultaneous determination of intracellular ATP concentration and rate of uridine uptake by quiescent and serum-activated 3T3 fibroblasts following treatment with 2-deoxyglucose. The cells were prepared and treated with 2-deoxyglucose (for 15 min) as described in Figs 1a and 2. The medium was changed to either 10% FCS in PBS for activated cells or PBS for quiescent cells, containing the appropriate concentration of 2-deoxyglucose and in addition $2 \text{ } \mu\text{Ci ml}^{-1}$ ^3H -uridine and $50 \text{ } \mu\text{M}$ unlabelled uridine and the cells incubated at 20°C for ~5–25 min. Uptake of uridine was terminated by washing five times with approximately 1 ml of ice-cold PBS and from each dish extracting both ATP and ^3H -labelled constituents together, using 1 ml of boiling water as described in Fig. 2. Aliquots of the water extract were taken for ATP assay as in Fig. 2 or for ^3H assay as in Fig. 1. a, ATP activity was measured and the data plotted for quiescent (○) and serum-activated (●) 3T3 fibroblasts as a function of 2-deoxyglucose concentration, for cells taking up uridine. Each point is the mean ($\pm$ standard error) of five determinations. b, Representative series of time courses of uridine uptake by the same cells shown in (a) treated with 0 mM (○, ●), 9 mM (□, ■) or 100 mM (△, ▲) 2-deoxyglucose; open symbols are quiescent cells, solid symbols serum-activated cells. The lines were obtained by linear regression analysis.

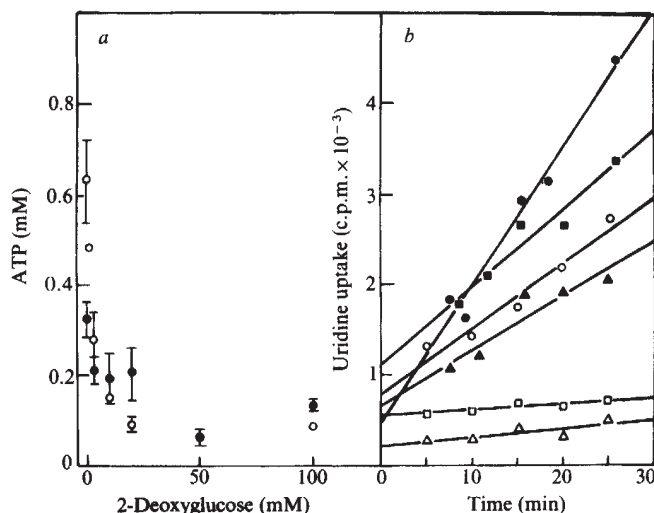
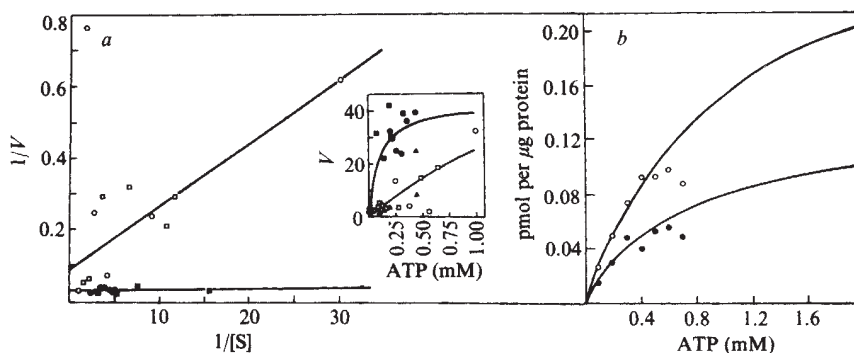


Fig. 4 a, Lineweaver-Burk plot of the reciprocal velocity (pmol per 10^6 cells per min) of uridine phosphorylation in quiescent and serum-activated 3T3 fibroblasts as a function of reciprocal intracellular ATP concentration (mM). The velocities were obtained from linear regression lines such as those shown in Fig. 3b. The ATP measurements, obtained as in Fig. 3a. The open symbols are quiescent cells, the closed symbols activated cells, circles and squares being separate experiments with serum as activator, the latter that of Fig. 3. The inset shows velocity of uridine phosphorylation in activated and quiescent 3T3 fibroblasts as a function of intracellular ATP concentration. The symbols are as described above except for the triangles, which represent an experiment in which 3T3 cells were treated with insulin 500 ng ml^{-1} (24.3 IU mg^{-1}) for 50 min ($\blacktriangle$) and control quiescent cells were not exposed to insulin ($\triangle$), using the same conditions of 2-deoxyglucose treatment described for serum-activation in Fig. 3. The lines drawn were calculated from the Michaelis-Menten equation using parameters obtained by a nonlinear least squares analysis (computer program NONLSQ, Hebrew University). **b**, Velocity of uridine phosphorylation using enzyme extracts from quiescent ($\circ$) or serum-activated ($\bullet$) 3T3 fibroblasts treated as described in Fig. 1a. After activation cells were scraped off the dishes with a rubber spatula, suspended in the residual medium and the cell membranes ruptured by repeated freeze-thawing (seven times) in liquid nitrogen. The enzyme assay was performed using $30 \mu\text{l}$ of enzyme solution, $10 \mu\text{l}$ of the appropriate concentration of ATP with equimolar MgCl_2 and $60 \mu\text{l}$ of 150 mM Tris buffer, $\text{pH } 7.4$ containing $50 \mu\text{M}$ uridine and $44 \mu\text{Ci ml}^{-1}$ ^3H -uridine. Incubation was at 37°C for 40 min and the reaction stopped in boiling water for 3 min. Radioactivity incorporated into nucleotide was determined by spotting $40 \mu\text{l}$ of the reaction mixture on Whatman DE 81 ion-exchange filter disks, drying under a hot lamp and washing away radioactivity not bound to the filter with approximately 100 ml of 1 mM ammonium formate. Total radioactivity on the disk was determined by spotting $5 \mu\text{l}$ of the mixture, drying but not washing away nonspecific activity with formate. The results are plotted as radioactivity incorporated into nucleotide in pmol per μg protein at each ATP concentration. Computer analysis of these data based on the Michaelis-Menten equation yielded the following kinetic parameters: for quiescent cell extracts the K_m was $1.0 \pm 0.1 \text{ mM}$ and the $V_{\max}$ $0.31 \pm 0.02 \text{ pmol per } \mu\text{g protein per min}$, and for activated cell extracts the K_m was $0.8 \pm 0.1 \text{ mM}$ and the $V_{\max}$ $0.14 \pm 0.02 \text{ pmol per } \mu\text{g protein per min}$.



no increase in $V_{\max}$ for uridine phosphorylation on serum stimulation; indeed, in this experiment a twofold decrease was observed. Thus, using enzyme extracts *in vitro*, we have been unable to demonstrate the enhanced affinity of the uridine phosphorylation system for ATP, which characterises the activated state *in situ*.

Our failure to reproduce *in vitro* the high ATP affinity for uridine phosphorylation in activated cells underlines the question as to the mechanism of activation *in situ*. It could be that the concentration of effectors of uridine kinase activity, such as UTP and CTP, might be depressed more in activated cells during the deoxyglucose treatment. Possibly in the extraction we have diluted either the enzyme or an allosteric effector needed to demonstrate the enhanced affinity, or perhaps reversed some covalent modification of the enzyme. Finally, preservation of anatomical integrity of the cell might be essential in order to demonstrate activation.

Operationally, activation of uridine uptake represents a dramatic increase in the apparent affinity for the substrate ATP in the *in situ* uridine phosphorylation system. The apparent twofold increase in $V_{\max}$, seen when uridine is the variable substrate (Fig. 1b), may be accounted for by the fact that such measurements were performed at an ATP level less than saturating for quiescent cells, but well above the K_m for activated cells (see Fig. 4a). The possible biological relevance of the change in K_m for ATP may be to ensure that uridine uptake can occur at a rapid rate, despite the drop in intracellular ATP concentration that we find occurs when activated cells take up uridine. The mechanism of this higher affinity for ATP in activated cells remains to be elucidated.

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T-cell lines producing antigen-specific suppressor factor

THE immune system is now recognised as a network of interacting cells with some interactions augmenting, and others suppressing immune responses. Regulatory interactions are often mediated by T cells, and do not always require cell-to-cell contact between appropriate cells, but may be mediated by soluble transmitters, commonly termed 'factors'. Both antigen-specific¹⁻⁷ and nonspecific⁸⁻¹⁰ factors have been described. Biochemical characterisation of these factors is hampered by the often minute quantities required to mediate biological effects and by the multiplicity of regulatory pathways involved in immune responses. Our knowledge of these factors is derived from functional tests, often combined with serological analysis of the factors. One approach to the analysis of monoclonal cell products is the somatic cell hybridisation technique described by Milstein *et al.*^{11,12}. Using this method, specific antibody-forming cells have been hybridised with myeloma cells, and have yielded clones producing specific antibody¹¹. This antibody is particularly useful as it is monoclonal and large quantities can be obtained. Although attempts to produce T-cell hybrids have been successful¹³⁻¹⁵, the products have not been functional. However, only failures in expression of cytotoxic activity have been described¹³⁻¹⁵. We describe here one antigen-specific suppressor factor (SF)-producing line S1-41 and its products.

Table 1 Specificity of suppression and molecular characterisation of S1-41 hybridoma factor SF S1-41

HC	Stimulus antigen	Suppression SF (%)	Response (anti-DNA AFC per culture)	% Suppression
Expt A—	TNP-KLH	—	33 ± 10*	—
HC _{KLH}	TNP-KLH	—	820 ± 92	0
HC _{KLH}	TNP-KLH	CBA SF _{KLH} 10	313 ± 19†	64
HC _{KLH}	TNP-KLH	SF S1.41 10	133 ± 39†	87
HC _{KLH}	TNP-KLH	SF S1.41 10	313 ± 24†	64
Expt B—	DNP-GAT	—	73 ± 12†	—
HC _{GAT}	DNP-GAT	—	383 ± 7	0
HC _{GAT}	DNP-GAT	SF S1.41 10	613 ± 56	0
HC _{GAT}	DNP-GAT	SF S1.41 1	493 ± 26	0
Expt C—	TNP-PAA	—	3,370 ± 82	0
—	TNP-PAA	SF S1.41 10	3,550 ± 262	0
—	TNP-PAA	SF S1.41 1	3,743 ± 471	0
Expt D—	TNP-KLH	—	90 ± 43†	—
HC _{KLH}	TNP-KLH	—	1,400 ± 178	0
HC _{KLH}	TNP-KLH	SF S1.41	333 ± 31†	81
HC _{KLH}	TNP-KLH	SF S1.41 abs. KLH	1,433 ± 129	0
HC _{KLH}	TNP-KLH	SF S1.41 elu. KLH	313 ± 29†	83
HC _{KLH}	TNP-KLH	SF S1.41 abs. GAT	260 ± 85†	87
HC _{KLH}	TNP-KLH	SF S1.41 elu. GAT	1,293 ± 24	8
HC _{KLH}	TNP-KLH	SF S1.41 abs. αIa ^k	1,613 ± 84	0
HC _{KLH}	TNP-KLH	SF S1.41 elu. αIa ^k	593 ± 81‡	62
HC _{KLH}	TNP-KLH	SF S1.41 abs. αIa ^s	493 ± 64†	71
HC _{KLH}	TNP-KLH	SF S1.41 elu. αIa ^s	1,513 ± 182	0
HC _{KLH}	TNP-KLH	SF S1.41 abs. αI-J ^k	1,480 ± 111	0
HC _{KLH}	TNP-KLH	SF S1.41 elu. αI-J ^k	307 ± 73†	83
HC _{KLH}	TNP-KLH	SF S1.41 abs. αI-J ^s	240 ± 84†	89
HC _{KLH}	TNP-KLH	SF S1.41 elu. αI-J ^s	1,267 ± 131	10
HC _{KLH}	TNP-KLH	SF S1.41 abs. αMig	300 ± 51†	84
HC _{KLH}	TNP-KLH	SF S1.41 elu. αMig	1,370 ± 278	2
HC _{KLH}	TNP-KLH	SF S1.41 +RαSF	1,520 ± 96	0
HC _{KLH}	TNP-KLH	SF S1.41 +MαSF	1,453 ± 152	0

For hybridisation, 10⁸ *in vitro* induced KLH-specific suppressor cells of CBA origin¹⁹, and 10⁷ BW5147 cells (a HGPRT-negative ACR thymoma line obtained from R. Hyman) were washed twice in serum-free balanced salt solution (BSS) and pelleted together at 400g. Poly-ethyleneglycol (0.5 ml; PEG, BDH, molecular weight 1,500) 50% in BSS, pH 7.8, was added drop by drop over 2 min as the cells were gently shaken into suspension. Serum-free BSS (0.5 ml) was added at the same rate and then drop by drop a further 5 ml BSS before slowly filling the tube to 20 ml with BSS. The cells were spun at 400g, the supernatant discarded and the cells resuspended in 100 ml Dulbecco's minimum essential medium (DMEM; Flow) with 20% fetal calf serum (FCS) and dispersed in 2-ml aliquots into 48 wells of Linbro trays (Flow). At 24 h after the fusion, 1.0 ml DMEM plus 20% FCS was removed and replaced by 1.0 ml HAT medium (DMEM plus 20% FCS containing 1.1 × 10⁻⁴ M hypoxanthine, 1.6 × 10⁻⁵ M thymidine and 4 × 10⁻⁷ M aminopterin) and this procedure was repeated on the next 2 d. On days 6, 8 and 10, HT medium was used (DMEM plus 20% FCS containing 1.1 × 10⁻⁴ M hypoxanthine and 1.6 × 10⁻⁵ M thymidine). Thereafter, (day 13 after fusion) the medium was changed to DMEM plus 10% FCS, and the contents of each Linbro well which started to grow within the next 1–2 weeks were subcultured in Linbro wells and then transferred to Nunc flasks (50 ml, Nunc/Delta 1461). Aliquots of supernatants were tested for function when the cells were growing in the Linbro plates, and subsequently in the flasks. For the assay, Marbrook-Diener cultures were incubated in a humidified atmosphere of 10% CO₂ in air at 37°C (ref. 20). Hybridoma supernatants were added to the mixture of KLH-, GAT- (copolymer of L-glutamine⁶⁰-L-alanine³⁰-L-tyrosine¹⁰- or (T,G)-A--L (poly (L-tyrosine-L-glutamine)-poly(DL-alanine)-poly(L-lysine)- primed helper cells (HC_{KLH}, HC_{GAT} or HC_{(T,G)-A--L} (primed *in vitro* as described previously¹⁹), normal spleen cells, and haptenated antigens TNP-KLH, DNP-GAT or DNP-(T,G)-A--L (ref. 18). The anti-DNP antibody-forming cells (AFC) were assayed on day 4 of the *in vitro* cooperative culture¹⁸. With unprimed B cells only IgM anti-DNP AFC were detected. All the cultures were carried out in triplicates and assayed separately. The assay is summarised as:

Spleen cells + haptenated antigen = background

Helper cells + spleen cells + haptenated antigen = help

Helper cells + spleen cells + haptenated antigen + suppressor cells S/N(SF) = suppression

Results are expressed as numbers of anti-DNP AFC per culture ± s.e. The degree of suppression was calculated as follows:

$$\% \text{ Suppression} = 100 - ([\text{suppression} - \text{background} / \text{help} - \text{background}] \times 100)$$

KLH-specific suppressor cells were induced by 4 d *in vitro* culture with 100 μg KLH ml⁻¹ (ref. 17), and fused with BW5147 cells on day 4 of culture. The medium used in the induction of helper or suppressor cells and in cooperative cultures was Eagle's minimum essential medium (MEM Gibco, with added bicarbonate 3.7 g l⁻¹ and 5% FCS) in the outside medium, and HEPES-buffered (4.36 g l⁻¹) MEM with 5% FCS in the inserts. *Expt A*, 3 × 10⁵ BIO.BR HC_{KLH} were added to 10⁷ normal BIO.BR spleen cells and the mixture was stimulated with 0.1 μg TNP-KLH ml⁻¹, SF were added to appropriate groups at the beginning of the 4-d cooperative culture. IgM anti-DNP AFC were assayed on day 4 of culture. The effect of SF S1.41 on KLH-specific help has been tested about 25 times over a period of 14 months. *Expt B*, lack of effect of SF S1.41 on helper cells of another specificity is shown by cultures containing 10⁵ BIO.BR HC_{GAT}, 10⁷ normal BIO.BR spleen cells and 1.0 μg DNP-GAT ml⁻¹. IgM anti-DNP AFC were assayed as in *expt A*. The specificity of SF S1.41 suppression has been tested on GAT or (T,G)-A--L-specific help four times over a period of 14 months. *Expt C*, lack of effect of SF S1.41 on a thymus-independent response is shown. 10⁷ normal BIO.BR spleen cells were stimulated with TNP-PAA (polyacrylamide beads) 0.3% final concentration in the absence or presence of SF S1.41. The assay of anti-DNP AFC was as in *expt A*. Two other experiments gave similar results. *Expt D*, 8 × 10⁵ BIO.BR HC_{KLH} were added to 10⁷ normal CBA spleen cells, and the mixture was stimulated with 0.1 μg TNP-KLH ml⁻¹ in the presence or absence of SF S1.41. The assay of anti-DNP AFC was as in *expt A*. For molecular characterisation of S-41 supernatant SF S1.41, the supernatant of S1.41 was absorbed and 'acid eluted' (using Sørensen's buffer pH 2.4) from the following immunoabsorbents: KLH (1 mg KLH/ml beads), GAT (1 mg GAT/ml beads), αIa^k (αIa^k serum was ATH anti-ATL 0.5 ml/5 ml beads), αI-J^k (BIO.HTT anti-BIO.S(9R) or BIO.A(3R) anti-BIO.A(5R) serum, 0.5 ml/5 ml beads), α-J^s (BIO.S(9R) anti-BIO.HTT serum, 0.5 ml/5 ml beads), and αMIG (0.5 ml rabbit anti-mouse Ig/5 ml beads). The beads used were Sepharose 4B (Pharmacia). The cyanogen bromide activation of beads and coupling of antigen or antisera was as described²². Preparation of anti-SF antisera and their characteristics are documented elsewhere¹⁷. They were used in cultures at 0.1% final concentration. Two other experiments of type D yielded similar results. Within each experiment the numbers of AFC in each group were compared to that of the positive controls (helper cells, normal spleen cells and antigen, second line in each experiment) using Student's *t*-test. *P* values are marked in the Table as follows: **P* < 0.001; †*P* < 0.01; ‡*P* < 0.05.

Because of our interest in antigen-specific soluble mediators of T-cell function, we decided to hybridise a thymoma line with *in vitro* induced antigen-specific suppressor T cells, known to release antigen-specific SF into the medium⁶. SF has been shown by the use of immunoabsorbents to have a combining site for antigen, Ia (I-J) coded determinants, no conventional Ig, but determinants reacting with anti-'constant' region and anti-'variable' region anti-SF antisera^{16,17} in the same molecule. SF acts on T helper cells of the same antigenic specificity, and shows no genetic restrictions in its function¹⁸. Two hybridisation experiments of this kind have been carried out so far and eight suppressor cell lines established. In the present experiment with cell line S1-41, a nonspecific suppressor cell line S1-34 and its product SF S1-34 was used as a control. Cell hybridisations were carried out essentially as described previously¹¹⁻¹³, using polyethyleneglycol.

In the first hybridisation 20% of the supernatants from Linbro wells containing growing cells showed suppressive activity. The majority of these suppressor hybridomas were later found to be contaminated with bacteria, but the remaining two, S1-41 and S1-34, were both of interest, one showing antigen-specific suppression (S1-41), the other nonspecific suppression (S1-34). S1-41 has now been cloned but the present report is of data obtained using supernatants from the uncloned line (SF S1-41). In the second hybridisation 20% of the hybridomas were initially selected as being suppressive; from these, six lines were established, some showing specific suppression and others nonspecific suppression. As shown in Table 1, SF S1-41 suppressed primary *in vitro* responses to the same extent as conventional suppressor factor CBA SF_{KLH} obtained from suppressor cells. This suppression is antigen specific, and does not affect help for responses of other antigenic specificity as shown on lines 6-9 of Table 1. Thymus-independent responses are also not affected, as shown by lines 10-12 of Table 1. The activity of SF S1-41 is absorbed out by the specific antigen (KLH), α Ia^k and α I-J^k, but not by an irrelevant non-cross-reactive antigen (GAT), α Ia^k, α I-J^k, or by rabbit antmouse immunoglobulin (α MIG) coupled to solid-phase immunoabsorbents, as shown in experiment D in Table 1. These results suggest that SF S1-41 carries an antigen-combining site and Ia(I-J)-coded determinants in the same molecule. Furthermore, the activity of SF S1-41 is abolished by rabbit or mouse (syngeneic) antisera to CBA SF_{KLH} (last two lines of experiment D, Table 1). Rabbit anti-SF (R α SF) inactivates all SF regardless of their strain of origin or specificity, and thus recognises 'constant' region-like determinants¹⁷. CBA anti-CBASF_{KLH}(M α SF) only inhibits the activity of CBA SF_{KLH} and thus recognises 'idiotype-like' determinants of the combining site of SF¹⁷. These results show that SF S1-41 carries both the 'constant' and 'variable' region determinants of

conventional suppressor factors; thus, SF S1-41 resembles conventional SF in all known aspects tested, including a molecular weight about 50,000-60,000 (refs 16, 17).

The effect of SF S1-41 on a secondary response is shown in Table 2, in which comparable suppression is obtained with a conventional SF and SF S1-41 on both IgM and IgG responses. For comparison, the effect of addition of SF S1-34 is shown. SF S1-34 is not antigen specific, and the molecule does not carry 'constant' region or 'idiotype-like' determinants (data not shown). The suppression by SF S1-41 and SF S1-34 has been tested repeatedly over a period of 14 months, with no change in suppressive capacity.

The Thy-1.2 (CBA parent) and Thy-1.1 (BW 5147) surface membrane phenotypes of the hybridomas were tested by direct cytotoxicity 1, 9 and 12 months after hybridisation. Ly-1, Ly-2 and Ly-6 phenotypes were tested by absorption 12-14 months after hybridisation. An example of data from the absorption analysis is shown in Fig. 1. The full phenotypes are as follows: S1-41, Thy-1.1⁺ Thy-1.2⁺ Ly-1.1⁺ Ly-1.2⁺ Ly-2.1⁻ Ly-6.2⁺, S1-34, Thy-1.1⁺ Thy-1.2⁺ Ly-1.1⁻ Ly-1.2⁻ Ly-2.1⁻ Ly-6.2⁺, BW, Thy-1.1⁺ Thy-1.2⁻ Ly-1.1⁻ Ly-1.2⁻ Ly-2.1⁻ Ly-6.2⁺. The Thy-1 phenotypes at least have been stable over 14 months.

Our results suggest that T-cell hybrids can be produced fairly readily when *in vitro* primed T cells, either T suppressor or T helper cells (unpublished) are used as a T-cell source. In the light of reported failures in obtaining functional T-cell hybrids¹³⁻¹⁵ the high frequency at which functional hybrids were obtained (20% of hybridomas being functional) may be surprising. It may be that T cells which have been recently exposed to antigen hybridise more readily, and after priming *in vitro* may adapt to further *in vitro* culture more easily.

The phenotypes of the hybrids are intriguing. S1-41 expresses both parental Thy-1 types (data not shown) confirming that it is a hybrid between AKR and CBA cells. Both parental Ly-1 alleles are also expressed. This is particularly interesting as our subline of BW5147 does not express Ly-1.2 (see Fig. 1). This suggests that although BW5147 has the structural gene for Ly-1.2, it has lost a gene controlling expression of the antigen. Presumably, this is provided by the CBA parent in the S1-41 hybrid. On the other hand, S1-34 fails to express either Ly-1 or Ly-2 of either parent.

Neither the phenotype of S1-41 nor S1-34 correspond to the phenotypes previously described for specific or nonspecific suppressors—respectively Ly-1⁻2⁺ and Ly-1⁺2⁺ (refs 23-25). It therefore seems that there is a dissociation between expression of Ly surface components and function in these two hybridomas. Alternatively, S1-41 may have some of the properties of suppressor cell inducers which interact with suppressor cell precursors, and are Ly 1⁺ (refs 25, 26).

Table 2 Effect of suppressor cell hybridoma S1-41 supernatant SF S1-41 on secondary antibody responses

Stimulus	Antigen	Suppression		Response			
		SF	Conc.(%)	IgM	(Anti-DNP AFC per culture $\pm$ s.e.)	IgG	(%S)
Spleen cells	—	—	—	67 $\pm$ 37*	(—)	87 $\pm$ 37*	(—)
CBA TNP-KLH 1°	—	—	—	2,300 $\pm$ 147	(0)	8,425 $\pm$ 485	(0)
CBA TNP-KLH 1°	TNP-KLH	—	—	1,617 $\pm$ 160†	(40)	5,067 $\pm$ 386†	(40)
CBA TNP-KLH 1°	TNP-KLH	CBA SF _{KLH}	10	1,000 $\pm$ 157†	(58)	7,050 $\pm$ 560	(16)
CBA TNP-KLH 1°	TNP-KLH	CBA SF _{KLH}	1	1,183 $\pm$ 177†	(50)	7,318 $\pm$ 1049	(13)
CBA TNP-KLH 1°	TNP-KLH	CBA SF _{KLH}	0.1	667 $\pm$ 100*	(73)	4,600 $\pm$ 83†	(46)
CBA TNP-KLH 1°	TNP-KLH	SF S1-41	10	950 $\pm$ 72†	(60)	6,683 $\pm$ 463	(30)
CBA TNP-KLH 1°	TNP-KLH	SF S1-41	1	1,483 $\pm$ 108‡	(37)	6,050 $\pm$ 707‡	(28)
CBA TNP-KLH 1°	TNP-KLH	SF S1-41	0.1	1,867 $\pm$ 254	(19)	3,867 $\pm$ 132*	(55)
CBA TNP-KLH 1°	TNP-KLH	SF S1-41	0.01	1,633 $\pm$ 201†	(30)	4,233 $\pm$ 372‡	(50)
CBA TNP-KLH 1°	TNP-KLH	SF S1-34	10	1,167 $\pm$ 174‡	(57)	4,117 $\pm$ 263*	(52)
CBA TNP-KLH 1°	TNP-KLH	SF S1-34	1	1,667 $\pm$ 204	(28)	4,195 $\pm$ 1095†	(51)
CBA TNP-KLH 1°	TNP-KLH	SF S1-34	0.1				

CBA mice primed with TNP-KLH on bentonite were boosted with 20 μ g TNP-KLH in saline intravenously 7 d before the cultures were started. 3×10^6 primed spleen cells were cultured in the presence or absence of SF. The stimulation was by 0.01 μ g ml⁻¹ TNP-KLH per culture¹⁶. IgM and IgG (developed by goat anti-IgM and sheep anti-IgG¹⁶) anti-DNP AFC were assayed on day 6 of culture. Percentage suppression (%S) is given in parenthesis. Three other experiments gave similar results. P values are marked as in Table 1.

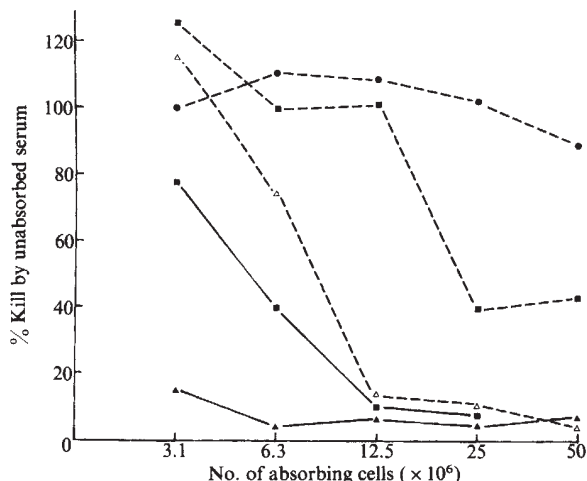


Fig. 1 Absorption analysis of the Ly surface membrane phenotype of hybrids. Preparation, testing and if necessary absorption of Ly sera and rabbit complement was as described previously²³. Sera were absorbed at the last dilution giving plateau lysis of appropriate thymocyte targets. Aliquots (50 μ l) of anti-Ly-1.1 (—) anti-Ly-1.2 (---), or anti-Ly-2.1 (data not shown) were absorbed for 1 h at room temperature with $3\text{--}50 \times 10^6$ tissue culture grown BW5147 (●), S1.41 (■), S1.34 (data not shown), or freshly prepared CBA (▲) or B10 thymocytes (△). Absorbed sera were assayed by a microcytotoxic test on CBA or B10 thymocytes. 2 μ l antiserum was added to 2 μ l cell suspension (2,000 cells in Hank's BSS + 5% FCS) in the wells of a microtitre plate (Falcon microtest 3034), and incubated at 37°C for 15 min. One drop of medium was then added and the plate refrigerated for 10 min. The supernatant was then removed by inverting and flicking the plate; 2 μ l 1/12 rabbit complement was added and the plate incubated at 37°C for 30 min. A drop of Trypan blue was then added, the plate again refrigerated and flicked, and a further drop of medium added for counting. For each antiserum, positive and negative control absorptions with appropriate thymocytes were carried out in each experiment. For simplicity, the negative control absorptions and negative results obtained with BW5147, S1.41 and S1.34 have been omitted from the figure, except for the example of anti-Ly-1.2 absorbed with BW5147. Other negative results showed essentially the same pattern.

The secreted suppressor factor of the S1-41 hybridoma, SF S1-41 showed no change in function or specificity over a period of 14 months. SF S1-41 has retained all the characteristics of conventional antigen-specific SF, carrying in the same molecule, antigen combining site, Ia (I-J) coded determinants and 'constant' region and 'idiotype-like' determinants of CBA SF_{KLH}. The latter are defined respectively by rabbit and mouse antisera raised against mouse SF¹⁷, and the determinants recognised by the sera have been termed 'constant' and 'idiotypic' or 'variable' by analogy with those parts of conventional Ig. It should be emphasised, however, that there is no evidence for the presence of conventional Ig determinants on either CBA SF_{KLH} (ref 16) or SF S1-41. Furthermore, S1-41 cells bear only T-cell markers Thy-1, Ly-1 and Ly-6, and do not carry surface Ig (unpublished). The characteristics of other specific and non-specific suppressor hybridomas and their products are under study as is the *in vivo* effect of the S1-41 hybridoma.

Our results show that functional T-cell hybrids can be produced by standard techniques and that the established hybrids are stable as judged by surface membrane phenotype and the functional properties of their secreted products. Such hybrids will be extremely useful in the more refined characterisation of the secreted products of T cells of various types, as unlimited cell numbers can be produced and the titre of SF produced by the hybrids is much higher than that of conventional factors.

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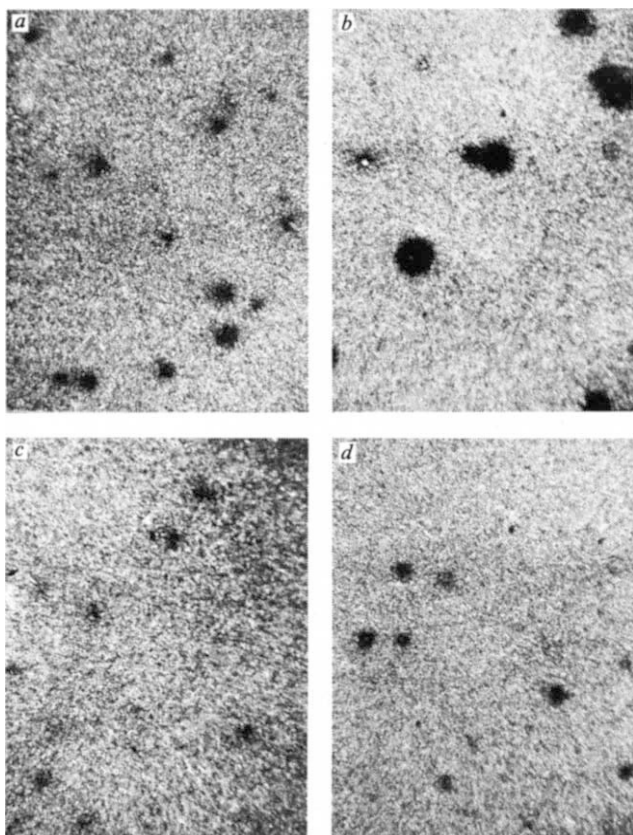
Most IgM-producing cells in the mouse secrete auto-antibodies (rheumatoid factor)

IN the mouse the injection of bacterial lipopolysaccharide (LPS) results in the appearance of very large numbers of IgM globulin-secreting cells¹. Furthermore, it has been shown that LPS induces B lymphocytes, both *in vivo*^{2,3} and *in vitro*⁴⁻⁶ to secrete IgM antibodies to a wide range of seemingly unrelated antigens. It was reported recently that about 8% of all IgM globulin-secreting cells arising as a consequence of LPS injection into CBA mice secrete an antibody-like molecule with specificity for a heterospecific (bovine) IgG (BIG) molecule¹. It was suggested that such IgM molecules were reminiscent of a rheumatoid factor, although at that time

plaque-forming cells (PFC) had not been demonstrated using mouse IgG (MIG) as the target antigen. I have now been able to demonstrate the presence of auto-(anti-MIG)-antibody-forming cells. About half (35–75%) of all IgM-secreting cells appearing after both mitogen and immunogen injection into mice produce IgM globulin which is not specific for the inducing immunogen, but which does have specificity for self-IgG. The description by Steel and Cunningham⁷ of a large proportion of IgM-PFC having specificity for normally hidden (syngeneic) erythrocyte antigens, supports a view that a majority of both mitogen and immunogen stimulated IgM-PFC are producing auto-antibodies. Although a mouse rheumatoid-like IgM with binding activity against antigen-bound (syngeneic) IgG was described previously⁸, until now attempts to develop a haemolytic plaque assay for autologous mouse M-anti-G have failed. After unsuccessful attempts involving various forms of mouse IgG, partially denatured mouse IgG and also a procedure used successfully in the rabbit⁹, a successful method has been developed. An account of some of the results from experiments using this methodology is presented here.

Figure 1 illustrates that mouse auto-immune (M-anti-G) haemolytic plaques are generally of a poorer quality than either reversed plaques (total IgM globulin-producing cells) or IgM anti-BIG plaques. Therefore anti-MIG plaques can only be counted accurately at a low concentration of less than about 50 PFC per slide, and most of the counts of these plaques are

Fig. 1 Photographs of reversed plaques (total IgM globulin) (a); direct plaques against TNP- (b); and indirect plaques against MIG (c) and BIG (d) 4 d after the intraperitoneal injection of 20 μ g LPS (*Salmonella typhosa* 0901 B). Although the anti-MIG plaques have a less complete haemolysis than the other plaques, microscopic examinations of dried glutaraldehyde fixed slides, stained with Wright, shows that all plaques have at least one lymphoid cell within them and most have only one which is unequivocally at the centre: this observation is facilitated by the very high frequency of anti-MIG PFC in the spleens of LPS injected mice.



probably underestimates and certainly relatively less than estimates for the other more clearly defined plaques.

After the injection of LPS into CBA mice there is a dramatic rise in PFC directed against a variety of antigens¹. In the context of the reversed plaques and the four target antigens used in the experiments described here, there are two distinct types of IgM—direct and indirect. The direct types are IgM antibodies specific for TNP- and for sheep red blood cells (SRBC) which appear on addition of guinea pig complement but do not require the presence of an anti- μ developing serum and the indirect types are anti-BIG, anti-MIG and reversed plaques which, in addition to a source of complement, require a rabbit anti-(mouse)- μ developing serum. In Fig. 2 it can be seen that the peak of the direct IgM response after LPS injection is on day 3 and for indirect IgM it is on day 4. It is not impossible that these operationally distinct types of IgM may reflect the presence of two sub-classes of murine IgM related in some, as yet undefined, way to the two sub-classes described by Plotz *et al.*¹².

The results shown in Fig. 3 show that anti-MIG plaques can be inhibited by free MIG at lower than the concentrations of free BIG previously reported for the equivalent LPS stimulated anti-BIG plaques¹. Perhaps a better comparison for the inhibition of anti-MIG plaques are the higher avidity plaques (inhibited by less free BIG) reported after immunisation with BIG + LPS: essentially these are PFC arising after immunisation with MIG + LPS. Figure 3 also shows that free BIG does not inhibit anti-MIG plaques, so it seems likely from this and the previously published data, that anti-BIG and anti-MIG-PFC are different populations of cells. The much greater inhibition of anti-MIG PFC by the Fc fragment of mouse IgG than by the Fab fragment, is consistent with those plaques being the result of the secretion of a rheumatoid factor-like molecule^{14,15}.

The reversed plaque assay used here allows an estimate to be made of the total number of cells synthesising IgM globulin. It is an excellent method for measuring the effectiveness of a very wide range of mitogens and furthermore it makes it possible to express both mitogen and immunogen stimulated responses as—that proportion of the elevated total IgM globulin production which is specific for a particular target antigen. Some recent experiments (unpublished data and ref. 16) with mice immunised with SRBC show that (1) less than 20% of the IgM-producing cells, which appear 4 d after antigen injection, can be assigned as having anti-SRBC specificity and (2) a large proportion of these IgM-producing cells are anti-MIG PFC: the degree of overlap, if any, between anti-SRBC and anti-MIG plaques is not known. Although there is no formal proof that anti-MIG specificity is due to a conventional antibody site, Metzger has argued convincingly that human M-anti-G specificity is due to such a conventional antibody activity¹⁷.

The existence of large numbers of auto-antibody-forming cells arising as a result of exposure to LPS or immunogens, in the presence of auto-antigen, suggests that this ability to manifest auto-immunity has arisen not as an accident, but as the consequence of particular selective pressures. The response directed against hidden auto-erythrocyte antigens or altered-self proteins may be a mechanism for the opsonisation of damaged or aged red blood cells and protein molecules, which assists their removal by macrophages from the blood¹⁸. Protection of cells against osmotic and mechanical stress has also been suggested as a function for auto-antibodies¹⁹. The possible role of an IgM response to bound or altered IgG (antibody) as a first step in the complement cascade has been proposed¹. As an extension of the hypothesis that auto-immunity can be interpreted as a break of tolerance²⁰ it has been suggested that large numbers of auto-reactive B-cells arise as the result of the elimination of specific regulatory T cells^{21–23}.

Since there is such a large capacity for synthesising and secreting IgM molecules which bind IgG (Fc) it is tempting to propose these molecules as candidates for a cybernetic network. However, the relative inability of Fab to inhibit

anti-MIG plaques (Fig. 3) makes it unlikely that this hypothetical network could have the fine specificity necessary for Jerne's proposed idiotypic-dependent network²⁴. Another possibility may lie in a role for auto-antigens in addition to environmental hetero-antigens, driving a mechanism for generating immune competence in B cells (immunological repertoire) in a manner analogous to that proposed for the role of MHC in driving the generation of diversity in T cells²⁵.

As stated above, the injection of an immunogen (SRBC) leads to an increase in total IgM globulin production, of which only a minor proportion can be assigned as having anti-SRBC specificity. If this is a general result of the injection of an immunogen and preliminary experiments suggest that this may well be so, then it could be imagined that the main difference

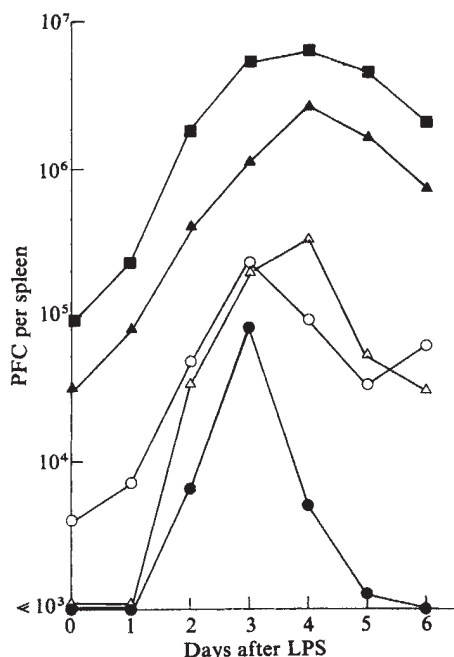


Fig. 2 The rise and fall in numbers of PFC after the intraperitoneal injection of 20 µg LPS into male CBA/Ca mice aged 10–20 months (lesser responses are obtained in younger mice), bred in an SPF unit and maintained in minimal disease conditions during experimentation. ■, Reversed-plaques (total IgM globulin); ▲, anti-MIG; and △, anti-BIG plaques; these three categories of plaque depend on the presence of a rabbit anti-(mouse) μ developing serum and consequently can be described as indirect μ plaques in contrast to direct μ plaques which do not require development. Two examples of direct plaques stimulated by LPS are included here, ○, anti-TNP and ●, anti-SRBC. The detailed methodology of Jerne's haemolytic plaque assay and of Molinaro and Dray's reversed plaque assay are described elsewhere¹⁰ together with an account of the cold chromic chloride method for coupling proteins to target erythrocytes. MIG was prepared by precipitation from mouse serum in half saturated ammonium sulphate, which was followed by purification on a column of Sepharose-(staphylococcal) protein A (Pharmacia) when the MIG was required for inhibition purposes. All classes of mouse IgG bind well to protein A in 0.01 M Tris-HCl pH 8.1 buffer and can be eluted in a pure state as judged by immunoelectrophoresis, using buffer at pH 2.8. MIG for coupling to target erythrocytes is further fractionated by precipitations in 25% ethanol at pH 6.5 (Cohn fraction II)¹¹. In addition to the titration of the rabbit anti-(mouse)- μ developing serum to find the optimal concentration, it was necessary to titrate the (MIG-Sepharose and SRBC absorbed) guinea pig complement against each batch of MIG-SRBC which was used: the optima lay between 1 and 5% in the present experiments. All lymphoid and target cells were kept in Hank's solution containing 0.5% Difco (Bacto) gelatine (HG). 'Day zero' mice did not receive LPS and these results can be considered as an indication of background levels.

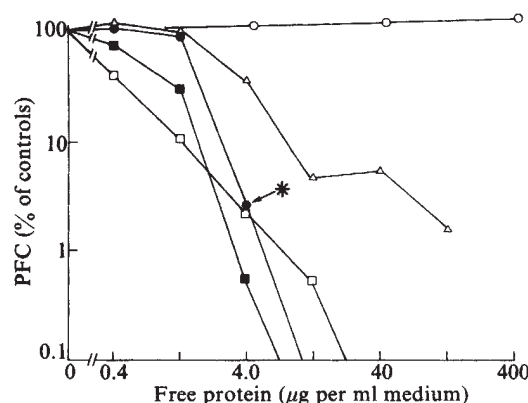


Fig. 3 The inhibition of day 4 post-LPS anti-MIG PFC by free proteins: MIG, ●; BIG, ○; mouse IgG-Fc, □; mouse IgG-Fab, △; and Fc + Fab, ■. The 100% values do not differ significantly from the day 4 values in Fig. 2. I thank G. G. B. Klaus for the Fc and Fab preparations, made by papain digestion and fractionation on Sepharose-protein A. Similar inhibition curves were obtained using Fc and Fab prepared by papain digestion and chromatography on DEAE-cellulose. MIG which had not been re-fractionated on Sepharose-protein A and which presumably contained traces of IgM, caused a general lysis of MIG-coated target cells. The observed degree of inhibition by Fab might be due to a small amount of contamination of the preparation by Fab or due to the exposure of normally hidden auto-antigenic Fab determinants similar to those described by Mandy *et al.* in the rabbit¹³. It is unlikely that the observed inhibition effect is due to the free-MIG interfering with the rabbit anti-(mouse)- μ developing serum, since a threefold increase in the concentration of developing serum (at the point marked *) has no effect on the number of detectable plaques. Passage of MIG down a column of Sepharose-(mouse)-IgM (TEPC183) did not result in a significant reduction in plaque number when this material was used as a target antigen. In contrast, reversed plaques were totally ablated, if an IgG preparation of goat anti-(mouse)- μ was passed down a similar column before being coupled to target erythrocytes. This result argues against a possibility that 'anti-MIG' plaques are due to a significant amount of anti- μ activity in the MIG preparations used to coat the target SRBC.

between a mitogen and an immunogen is that the former has a random effect in binding to any and all cells, whereas the immunogen is concentrated on those lymphocytes with receptors for antigen: these may be IgD in virgin precursor cells and Ig of the class eventually secreted in memory cells¹⁵. This mechanism is superficially similar to the hypothetical mechanism proposed by Möller⁵, who suggests that the role of antigen-receptors is to concentrate antigen (mitogen) near the mitogen-receptor which triggers the lymphocyte to differentiate into a cell secreting IgM. However, it is unlikely that this is a general mechanism since completely separate, nonimmunogenic antigen and adjuvant molecules can successfully trigger an IgG response²⁶.

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High proportion of Ig-producing cells making autoantibody in normal mice

'NATURAL' serum antibodies are thought to arise as a result of stimulation by environmental antigens. Evidence suggests, however, that many such antibodies in normal healthy individuals are specific for and may be induced by a variety of self components. Many of these autoantibodies are directed against 'buried' or 'enzyme-revealed' self antigens and include those specific for mouse reproductive organs which crossreact with human group A erythrocytes¹, human and rabbit immunoglobulins treated with proteolytic enzymes^{2,3}, denatured DNA⁴, enzyme-treated human lymphocytes and erythrocytes^{5–8}, and mouse erythrocytes treated with the proteolytic enzyme bromelain⁹. This widespread occurrence of autoantibodies could suggest that the normal immune system is preoccupied in reacting against self antigens. One way of measuring the extent of this reactivity is to compare the number of B cells making a given autoantibody(s) with the total number of B cells producing immunoglobulin (Ig) irrespective of specificity. We have previously shown that normal conventional and germ-free mice possess in their spleens considerable numbers of PFC against bromelain-treated isologous mouse erythrocytes (BrM), an 'internal' antigen of mouse erythrocytes^{9,10}. We have measured the proportion of Ig-secreting cells in the lymphoid organs of normal CBA/H mice forming PFC against BrM and found that in some (but not all) major lymphoid organs between 1% and >50% of the existing or potential Ig-secreting cells are specific for BrM.

All PFC assays were carried out using the liquid monolayer system of Cunningham and Szenberg¹¹. Bromelain-treated mouse erythrocytes were used to detect BrM PFC (ref. 10). Ig-secreting cells (Ig PFC) were detected by the reverse plaque method¹² using sheep erythrocytes coated with the globulin fraction of a polyvalent hyperimmune sheep anti-mouse Ig serum by the chromium chloride method¹³. Reverse plaques require the presence of a rabbit anti-mouse Ig-developing serum at about the same concentration as used to detect conventional specific indirect plaques (see ref. 14). The detection system is polyvalent, revealing B cells producing IgM, IgG and IgA (data not shown). The direct BrM PFC are formed by cells making IgM antibody. Thus, the 'true' proportion of BrM PFC to total IgM PFC will be greater than the frequencies reported here if significant numbers of cells secreting Ig classes other than IgM are present in a given organ.

The numbers of BrM PFC and Ig PFC in various organs of CBA/H mice are shown in Tables 1 and 2. Table 1 displays the 'natural' antibody producers in freshly collected cell suspensions, whereas Table 2 shows results for cells cultured for several days in the presence of lipopolysaccharide (LPS)—a polyclonal B cell activator¹⁵—to identify 'potential' antibody producers.

The spleens of normal male or female CBA/H mice contain about 5,700 Ig PFC per 10⁶ cells (Table 1). Ig PFC are also found in other lymphoid organs, notably mesenteric lymph nodes (3,000/10⁶), bone marrow (1,030/10⁶), thymus (310/10⁶) and Peyer's patches (15,000/10⁶). Few are present in popliteal and parathymic lymph nodes or in freshly collected peritoneal or pleural cavity cells. BrM PFC have a different distribution. Expressed as a percentage of Ig PFC, BrM PFC are high in spleen (1.6%) and bone marrow (7.4%), low in thymus (0.6%) and mesenteric lymph nodes (0.2%) and undetectable in Peyer's patches (<0.01%).

If peritoneal cells are cultured *in vitro* they can be shown to rapidly produce many BrM PFC (refs 16 and 17). It has been shown that the appearance of BrM PFC in peritoneal (and spleen) cell cultures requires little cell division but does depend on RNA and protein synthesis¹⁶. The mechanism of the normal *in situ* suppression of these potential PFC is not clear, although there is some evidence that an antigen dependent blocking process is involved¹⁸.

The large number of BrM PFC generated in short-term peritoneal cell cultures suggested that much of the Ig-secreting cell potential in this site may be directed against BrM. Table 2 summarises results of a series of experiments comparing the proportions of BrM and Ig PFC after short-term culture in the presence of LPS. For spleen 1–2% of Ig PFC were BrM specific (same as before culture, Table 1). Peyer's patch cells, containing few BrM PFC before culture (Table 1), also show very low numbers after culture. Parathymic lymph nodes behave similarly in this respect (data not shown). The most striking result occurs in cultures of peritoneal or pleural cavity cells, where between 40% and 95% of the Ig PFC are BrM specific. It is noted that older mice, particularly retired breeder females contain more potential PFC than younger mice. Lord and Dutton¹⁶ have reported no increase with age in the number of BrM PFC generated by peritoneal cells *in vitro*. Our findings agree, however, with earlier studies by Nossal *et al.*¹⁹ who showed that peritoneal cell cultures from retired breeder females produce many more PFC against sheep erythrocytes—an antigen known to crossreact with BrM^{17,18}—than young male mice.

In other experiments (data not shown) we have shown that removing cells forming rosettes with BrM (1% in spleen, 20%

Table 1 Tissue distribution of Ig-secreting cells and BrM PFC in CBA/H mice*

	PFC per 10 ⁶ cells		
	Ig	BrM	%BrM†
Spleen	5,700 ± 350‡	87 ± 17‡	1.6 ± 0.4‡
Thymus	310 ± 150	1.4 ± 1.1	0.6 ± 0.2
Parathymic lymph nodes	180 ± 48	5 ± 3	2.2 ± 1.2
Mesenteric lymph nodes	3,000 ± 830	7 ± 4	0.2 ± 0.1
Popliteal lymph nodes	38 ± 21	≤ 14	
Bone marrow	1,030 ± 370	70 ± 22	7.4 ± 0.7
Peyer's patches	15,000 ± 3,400	< 1	< 0.01
Peritoneal cells	250 ± 190	42 ± 24	15 ± 11
Pleural cavity cells	< 50	< 50	

* Results summarised from four experiments using normal male (two experiments) and female (two experiments) CBA/H mice (age range 1.5–5 months). In each experiment pooled tissues from four age- and sex-matched mice were used. Viable nucleated cells were determined by trypan blue exclusion. The average viable cell counts per organ ± s.e.m. (× 10⁶) were: spleen (62 ± 11), thymus (129 ± 40), parathymic lymph nodes (4.5 ± 0.8), mesenteric lymph nodes (13 ± 0.6), popliteal lymph nodes (0.9 ± 0.25), bone marrow (16 ± 2.3, per tibia and femur), Peyer's patches (0.9 ± 0.2), peritoneal cells (2.6 ± 0.5), and pleural cavity cells (1.1 ± 0.1).

† %BrM = (BrM PFC/Ig PFC) × 100.

‡ Arithmetic mean ± s.e.m. for four experiments.

Table 2 Proportion of Ig-secreting cells making BrM antibody after short-term culture*

Experiment no.	Days in culture		PFC per 10 ⁶ original cultured cells		% BrM†
			Ig	BrM	
1. 6 week (female)	3	Spleen	65,000‡	500‡	0.8
		Peritoneal cells	15,000	6,000	40.0
2. 11 week (female)	2	Spleen	36,900	580	1.6
		Peritoneal cells	21,000	15,900	75.4
3. 14 week (female)	3	Peritoneal cells	28,200	24,400	86.5
		Pleural cells	25,600	16,000	66.4
4. Retired breeder female	2	Peritoneal cells	39,140	37,060	94.7
5. Retired breeder female	3	Spleen	105,600	2,040	1.9
		Peyer's patches	20,800	34	0.16

* Cells from CBA/H mice were cultured for several days in polyacrylamide dimple rafts²². Each raft contained 1–2 × 10⁶ viable cells, 10 µg LPS (from *E. coli* 0128:B12) in Eagles' minimal essential medium containing 10% fetal calf serum and 10⁻⁴ M mercaptoethanol. For serous cavity cells high numbers of Ig and BrM PFC were evident at least by day 1 (data not shown).

† % BrM = (BrM PFC/Ig PFC) × 100.

‡ Mean PFC counts on duplicate or triplicate cultures. Percentage s.e. between cultures was ≤ 15%.

in peritoneal cells) before culture abolishes the appearance of BrM PFC. Although this has little effect on Ig PFC in spleen, it causes a proportional or total removal of Ig PFC from peritoneal cell cultures. This result is consistent with the relatively low proportion of potential BrM PFC in spleen and the high proportion of potential BrM PFC in peritoneal cell populations.

It is clear from the present work that a high proportion of Ig-secreting cells in normal mice are specific for BrM. Several lines of evidence indicate that this result is probably not unique. First, there are many reports showing that the incidence of autoantibodies is widespread amongst normal healthy individuals^{1–10}. Second, Dresser²⁰ has recently claimed that a high proportion of IgM-secreting cells in mouse spleen (~50%) are specific for antigens on isologous IgG. Third, it has been known for some time that a large percentage of Waldenstrom macroglobulins (10–20%) possess auto-specificity for human IgG (reviewed by Metzger²¹). These observations suggest that much of the 'natural' Ig synthesis of an animal (existing and potential) may be directed towards endogenous antigens. If this hypothesis is correct it has far-reaching implications on the way we view the normal development and functioning of the immune system.

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Identification of ataxia telangiectasia heterozygotes, a cancer prone population

In ataxia telangiectasia (AT), an autosomal recessive disease of man with neurological, cutaneous and immunological abnormalities¹, cells from homozygotes are sensitive to ionising radiation² and have a low capacity to repair the resulting DNA damage³. Heterozygotes lack the main clinical features of the syndrome but share with homozygotes a high predisposition to cancer⁴. One study has demonstrated an increased level of chromosomal abnormalities in some heterozygotes⁵. Because these heterozygotes represent ≥1% of the population⁴ and cannot be diagnosed clinically, their characterisation represents a major challenge in cancer epidemiology. We report here the laboratory identification of a series of obligate heterozygotes for AT, based on the sensitivity of lymphoblastoid cell lines to ionising radiation.

Peripheral blood lymphocytes were isolated⁶ from five AT homozygotes, eight obligate heterozygotes, one clinically normal sibling of an AT homozygote and 11 controls. One aliquot of lymphocytes was transformed with Epstein-Barr

Table 1 DNA replication and repair in PHA-stimulated lymphocytes

	DNA replication (c.p.m. × 10 ⁻⁵ per 10 ⁶ cells)	DNA repair (c.p.m. × 10 ⁻³ per 10 ⁶ cells)	Normalised* repair
Controls	4.4 ± 2.7	6.1 ± 0.8	0.014
AT heterozygotes	5.8 ± 3.1	7.4 ± 3.2	0.013
AT homozygotes	2.7 ± 0.7	1.0 ± 0.2	0.004

Peripheral blood lymphocytes from 11 controls, eight heterozygotes and five homozygotes were separated by sedimentation on methyl cellulose⁶, and plated in medium (F15, Gibco) supplemented with 15% fetal calf serum, penicillin (10⁵ U l⁻¹), streptomycin (60 mg l⁻¹), and 1% PHA (v/v, Gibco). DNA replication was determined on day 4 after addition of PHA by measurement of incorporation of ³H-thymidine (Amersham, 25 Ci mmol⁻¹, 5 µCi ml⁻¹) into DNA precipitated with 5% trichloroacetic acid and filtered. DNA repair was determined at the same time by irradiating cells in a Gamma Cell 220 (Atomic Energy of Canada Ltd) with 40 krad, at 6.5 krad min⁻¹. Repair synthesis was measured in the presence of 10 mM hydroxyurea as described previously¹⁰. Replication and repair are given as mean ± s.d. Homozygotes used in this study have been designated AT1ABR through AT5ABR. This notation replaces AT1–AT4 which was used previously for the first four AT homozygotes¹¹. The replication value for AT3ABR (AT3), which is approximately an order of magnitude lower than that for the other AT homozygotes¹¹, has not been included.

* Normalised repair refers to the ratio of DNA repair to DNA replication when the latter is normalised to 5 × 10⁵ c.p.m. This ratio has been found to remain constant for a given lymphocyte population from day 3 to day 5 after addition of PHA (unpublished observations).

virus (EBV)⁷ to produce lymphoblastoid cell lines. These were used to determine cell viability by trypan blue exclusion⁸ and to determine cell survival by colony formation in agar⁹, after γ irradiation. A second aliquot was stimulated by phytohaemagglutinin (PHA) before determination of DNA repair synthesis following γ irradiation, with hydroxyurea used to inhibit DNA replication¹⁰.

Lymphocytes from AT homozygotes showed a much lower DNA repair capacity compared with controls (Table 1), as described in detail elsewhere¹¹, in keeping with data from some AT fibroblasts³. However, this assay did not distinguish AT heterozygotes from controls (Table 1).

Lymphoblastoid cell lines have been shown to reflect the ultraviolet radiosensitivity exhibited by fibroblasts from the same individuals in the case of xeroderma pigmentosum⁸. In our experiments radiosensitivity determined by colony formation in agar of γ -irradiated lymphoblastoid cells was different in the three groups (Fig. 1). The D_0 value for heterozygotes was 80 ± 7.4 in comparison with a control value 101 ± 11.6 ($P <$

Fig. 1 Effect of γ radiation on survival of lymphoblastoid cells in agar. Mean survival values for: ●, four control lymphoblastoid cell lines; ○, six AT heterozygotes; ■, five AT homozygotes. Lines were fitted by least squares linear regression analysis to the points shown and significance was established using Student's t test. Each point plotted represents the mean value of a series of measurements on the cells from a given individual. Cultures were maintained in suspension in RPMI medium (1640, Gibco) supplemented with 20% fetal calf serum. Cells were suspended in appropriate concentrations in agar droplets immersed in medium⁹, before irradiation in oxalic conditions at 600 rad min^{-1} . Cells were incubated at 37°C in 5% CO_2 for 12–14 d with a change of medium after 7 d. After colony formation agar droplets were washed in 0.9% saline, fixed with methanol and allowed to dehydrate before staining with Giemsa. Colonies containing ≥ 50 cells were scored. The cloning efficiency for controls and heterozygotes was approximately 2% whereas that for homozygotes was 0.5%. This variation required appropriate adjustments of cell numbers.

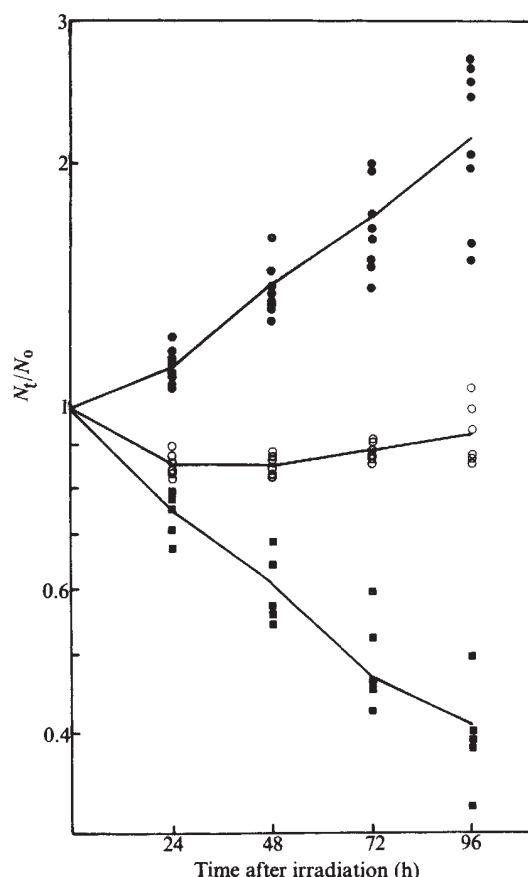
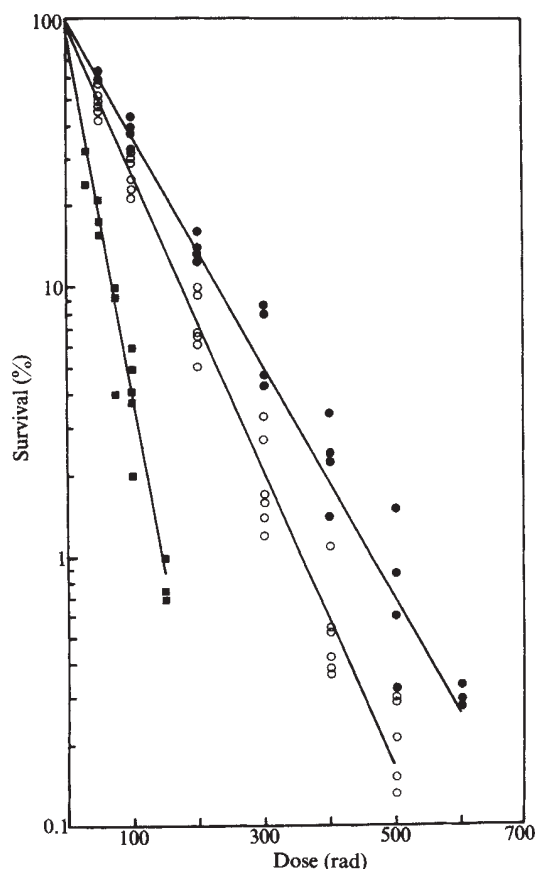


Fig. 2 Effect of γ radiation on lymphoblastoid cell viability. Cell viability was determined by trypan blue exclusion⁸. ●, Eight control lymphoblastoid cell lines; ○, six AT heterozygotes; ■, five AT homozygotes. Cells were plated out at $2 \times 10^5 \text{ ml}^{-1}$ in 36-mm dishes and irradiated with 400 rad. Cultures were then incubated in an atmosphere of 5% CO_2 . Cell viability was determined at daily intervals after irradiation by adding 0.1 ml of 0.4% trypan blue to a 0.5-ml cell suspension before counting. Significance was established using Student's t test. Each point plotted represents the mean value of a series of measurements on the cells from a given individual. N_0 , viable cell number at time 0; N_t , viable cell number at given time.

0.001). The homozygote value of 36 ± 3.8 was significantly lower than that for heterozygotes ($P < 0.001$); this greater sensitivity of AT lymphoblastoid cells to ionising radiation is similar to that for AT fibroblasts². Cell survival as determined by cloning in agar thus provides a means of distinguishing AT heterozygotes from controls and from homozygotes.

This method of assay is somewhat slow and tedious; measurement of viability of the lymphoblastoid cells in suspension by dye exclusion provides a more rapid assay system. The results in Fig. 2 show a trimodal distribution when viability was determined after exposure to a standard dose of γ -radiation (400 rad). Control cells continued to divide, while homozygote cell numbers decreased, reflecting the increased sensitivity of the latter seen in the studies of colony-forming ability (Fig. 1). AT heterozygotes could be distinguished readily from controls and homozygotes: at the dose of radiation used there was little or no change in viable cell number up to 96 h after γ irradiation. Quantitative comparison of heterozygotes with controls, and of heterozygotes with homozygotes, showed these differences to be statistically significant ($P < 0.001$) 24, 48, 72 and 96 h after γ irradiation. The standard radiation dose of 400 rad was chosen to maximise the distinction between the responses of the three cell groups, on the basis of preliminary experiments with a range of doses.

In both assays with lymphoblastoid cells, a clinically normal sibling of the homozygote AT3ABR showed responses in the heterozygote range. Because the highly significant survival data for obligate heterozygotes clearly indicate that carriers of AT mutations can be detected by this means, we have tentatively designated this sibling as a heterozygote. Extensive family studies are obviously required. However, in view of the likely existence of several complementation groups¹², all heterozygotes may not fit into the same pattern: possibly the present mutants belong by chance in the same complementation group. Conversely, it is predictable that not all heterozygous mutants showing a similar pattern will fall into the AT class as designated by the homozygote clinical presentation, that is the assays cannot be expected to be truly specific in their present form.

On the basis of an incidence of 1/40,000 for AT homozygotes⁴, the predicted heterozygote frequency would be 1%. However, if there is a number of complementation groups, the true heterozygote frequency could be substantially higher. Identification of heterozygotes thus becomes potentially important in numerical terms in preventive medicine, in view of the associated high cancer risk⁴. A high cancer risk is also seen in heterozygotes for Fanconi's anaemia¹³, another autosomal recessive syndrome with anomalies in DNA repair¹⁴. The fact that AT heterozygotes share this risk with homozygotes and that an abnormality can be detected in heterozygous lymphoblastoid cells should also be helpful in elucidating the minimal effects required for association between this class of mutations and carcinogenesis. The finding by Paterson *et al.* (personal communication) that the fibroblasts of some AT heterozygotes have radiosensitivity intermediate between controls and homozygotes strengthens the view that this is indeed a heterozygote characteristic.

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A vitamin A analogue inhibits radiation-induced oncogenic transformation

The ability of vitamin A and its analogues (retinoids) to prevent chemically-induced tumours has been studied in various experimental systems¹. We have investigated here the possibility that retinoids might also inhibit radiation-induced oncogenic transformation using a cell line of C3H mouse embryo fibroblasts grown in culture. We report a marked reduction in the transformation frequency by using one such compound, Ro-11-1430, the synthesis and structure of which has been described by Bollag².

C3H 10T_{1/2} clone 2 cells, when treated with polycyclic hydrocarbons, produced morphologically and malignantly transformed foci with a dose-dependent frequency. Cloned foci could give rise to fibrosarcomas when inoculated into irradiated syngeneic mice^{3,4}. The same cell line has been studied by Terzaghi and Little, who observed X-ray induced oncogenic transformation and found that the number of transformants per viable cell increased exponentially with dose up to about 400 rad (ref. 5). These findings have been reproduced and extended in our laboratory⁶. The quantitation of oncogenic transformation assays *in vitro* has been previously described^{7,8}.

The cells used in our experiments were between passages 13 and 15 because such cells are characterised by a very low incidence of spontaneous transformation. For this first series of experiments the trimethylmethoxyphenyl analogue of *N*-ethylretinamide (Ro-11-1430) was selected because it has been reported to be effective in different experimental systems without being toxic to the host^{2,9,10}.

Four replicate experiments were carried out in which the transformation frequency following a single dose of ⁶⁰Co γ rays was determined with or without the addition of the retinoid Ro-11-1430 at a concentration of 7.1 μ M. The results of these experiments are summarised in Table 1. The drug alone produced no toxicity at the concentration used. However, the transformation rate was significantly reduced when the retinoid was present, the number of transformants per surviving cell being reduced from $9.04 \pm 0.47 \times 10^{-4}$ to $2.67 \pm 0.48 \times 10^{-4}$.

Preliminary experiments indicate that at much higher drug concentrations the transformation frequency is reduced to zero. However, at these higher concentrations, some toxicity was apparent and complicates the interpretation of the data. Further experiments with other vitamin A analogues are in progress and indicate that the ability to inhibit radiation-induced transformation is not confined to Ro-11-1430.

It is worth noting that the results of the present experiments were obtained by using mouse fibroblasts as opposed to the epithelial cells; therefore, the action of retinoids does not seem to be confined to epithelial tissues. Indeed, it has been reported that these compounds are also active against mesenchymal tumours, as shown by the inhibition of growth and the regression of established transplantable chondrosarcoma in rats⁹. The ability of vitamin A to protect laboratory animals from developing chemically-induced neoplasms has been documented in various laboratories¹¹⁻¹³. Because of inadequate tissue distribution or excessive toxicity, natural retinoids have been judged to be of limited usefulness as agents for the prevention of cancer in either animals or man¹. Current research is aimed at developing synthetic analogues of vitamin A which are more potent and at the same time less toxic; such synthetic compounds have been evaluated in animal models^{2,10,14} and various organ systems in culture¹⁵⁻¹⁸ for their ability to prevent chemically-induced epithelial metaplasia or neoplasia.

It has been proposed that retinoids should be administered to high-risk populations for the prevention of neoplasms for

Table 1 Oncogenic transformation by ^{60}Co γ rays with and without the vitamin A analogue Ro-11-1430

Treatment	Expt no.	No. of surviving cells per dish	No. of dishes	PE (%)*	SF†	No. of transformations		Total no. of transformed foci/total surviving cells	Average frequency of transformation $\times 10^{-4}$ ($\pm$ s.e.)
						Type II	Type III		
Controls (no radiation no drug)	1	196	22	10	1.00	0	0	$0/4.31 \times 10^3$	0
	2	200	22	5	1.00	0	0	$0/4.40 \times 10^3$	
	3	272	22	14	1.00	0	0	$0/5.98 \times 10^3$	
	4	550	15	27	1.00	0	0	$0/8.25 \times 10^3$	
7.1 μM Ro-11-1430	1	191	44	—	0.97	0	0	$0/8.4 \times 10^3$	0
	2	256	35	—	0.95	0	0	$0/8.96 \times 10^3$	
	3	272	45	—	1.00	0	0	$0/1.22 \times 10^4$	
	4	540	10	—	0.98	0	0	$0/5.40 \times 10^3$	
400 rad γ rays	1	181	40	—	0.15	1	6	$7/7.24 \times 10^3$	8.63 ± 0.53
	2	240	51	—	0.40	4	6	$10/1.22 \times 10^4$	
	3	423	44	—	0.31	3	13	$16/1.86 \times 10^4$	
	4	412	36	—	0.30	3	8	$11/1.48 \times 10^4$	
400 rad γ rays + 7.1 μM Ro-11-1430	1	276	90	—	0.14	0	9	$9/2.48 \times 10^4$	2.46 ± 0.40
	2	234	99	—	0.39	2	3	$5/2.32 \times 10^4$	
	3	432	105	—	0.32	0	10	$10/4.54 \times 10^4$	
	4	412	40	—	0.30	2	1	$3/1.65 \times 10^4$	

C3H 10T $\frac{1}{2}$ cells between passages 13 and 15 were used, as their spontaneous transformation rate is undetectably low. The cells were grown in Eagle's basal medium (MBE), supplemented with 10% heat-inactivated fetal calf serum, penicillin (50 units ml^{-1}) and streptomycin (50 $\mu\text{g ml}^{-1}$). Appropriate numbers of cells were seeded into 100-mm Falcon plastic Petri dishes so that about 400 cells per dish survived the various experimental treatments. The drug dissolved in ethanol was added to the culture medium at the time the cells were seeded, to a final concentration of 1.75×10^{-5} . Irradiations were carried out 24 h later using a ^{60}Co teletherapy unit, at a distance of 80 cm the dose rate was $91.9 \text{ rad min}^{-1}$. The drug was left in contact with the cells for a total period of 96 h, after which it was removed and fresh (drug free) medium added to the cells. All steps in the experimental procedure which involved the drug were carried out in subdued light. Following the completion of all treatments with drug and radiation, the cells were incubated at 37.5°C , and the culture medium was changed weekly, for a period of five weeks. By this time, the untransformed cells had formed a confluent monolayer, and transformed clones were large enough to be easily visible and readily distinguishable by their morphology. Transformed foci were evaluated using morphological criteria outlined by Reznikoff *et al.*^{3,4} In the present experiments, only type 3 foci were scored as transformants; these are very densely piled up stellate cells with pronounced criss-crossing and swirling of cells at the border of the clone. No transformations were observed in unirradiated cells, regardless of whether or not the retinoid Ro-11-1430 was present. This indicated that the spontaneous transformation, if it occurs, is exceedingly rare.

*PE = Plating efficiency = no. of clones counted/no. of cells seeded.

†SF = Surviving fraction = no. of clones surviving drug and/or radiation/(no. of cells seeded $\times$ PE).

which chemical carcinogens are implicated. Our findings suggest that these compounds could also have a role in the prevention of neoplasms for which radiation may be a contributing factor.

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Glycosylation is not necessary for membrane insertion and cleavage of Semliki Forest virus membrane proteins

RECENT translation studies *in vitro*^{1,2,33} have shown that the synthesis of membrane ectoproteins, like the glycoproteins of vesicular stomatitis virus (VSV) and Semliki Forest virus (SFV), (budding, enveloped viruses), occurs simultaneously with their insertion into membranes, their glycosylation and their proteolytic processing. We have studied the synthesis of SFV membrane proteins in infected cells treated with tunicamycin, which prevents glycosylation of proteins by inhibiting the formation of the lipid bound sugar intermediates needed for protein glycosylation³⁻⁵. We have found that glycosylation is not a prerequisite for correct insertion and cleavage of such membrane ectoproteins.

In cells infected with SFV all the structural proteins are read sequentially from one 26S RNA using one initiation site⁶⁻⁹ in the order capsid protein C (molecular weight 30,000; ref. 10) and the membrane glycoproteins p62 (62,000) and E1 (49,000; ref. 11). The p62 protein is a precursor¹² of the viral glycoproteins E2 (52,000) and E3 (10,000; ref. 11). During translation of the 26S mRNA the C protein is removed from the nascent chain, and the newly exposed NH_2 terminus directs the ribosome to the membrane of the endoplasmic reticulum (ER) where synthesis and insertion of first p62 and then E1 takes place (H.G. *et al.*, unpublished). The major parts of the two membrane proteins including their NH_2 termini are located in the cisternal space of the ER, whereas their COOH termini

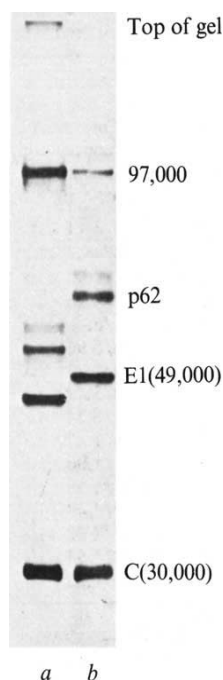


Fig. 1 Viral proteins present in control and tunicamycin-treated cells. Monolayer cultures of BHK cells were infected with SFV in medium lacking methionine²⁸. After 2 h tunicamycin was added to some of the cultures ($0.75 \mu\text{g ml}^{-1}$)¹⁶, the rest remaining as controls. About 4 h after infection the cells were pulsed with ^{35}S -methionine ($30 \mu\text{Ci ml}^{-1}$) for 10 min. The cells were solubilised in 2% SDS and equal samples, containing approximately 4×10^4 c.p.m. ^{35}S -methionine in the viral proteins, were analysed on a 10% SDS gel²⁹. The labelled proteins were visualised by fluorography³⁰. Approximate molecular weights of unknown protein bands were calculated from their position relative to those of the known proteins. About the same amount of ^{35}S -methionine-labelled viral protein was made during the pulse in tunicamycin-treated cells as in control cells. *a*, Viral proteins made in tunicamycin-treated cells; and *b*, viral proteins made in control cells.

attach these proteins to the membrane³⁴. The COOH terminus of p62 spans the membrane there being about 30 amino acids at the cytoplasmic side of the Er membrane²⁰. A small fraction of the membrane proteins made in the cell fail to be inserted and remain outside the Er membrane as an uncleaved and non-glycosylated 97K protein.

Figure 1 shows the viral polypeptides made in control and in tunicamycin-treated cells. Tunicamycin treatment should only change the migration behaviour of such proteins that are normally glycosylated. Figure 1*a* shows that the normally non-glycosylated C and 97K proteins are present in tunicamycin-treated cells, whereas no bands corresponding to the glycosylated protein species p62 (a double band) and E1 are seen^{13,14}. Instead, aberrant proteins with apparent molecular weights of 54,000 (a double band) and 47,000 are present. The extent of glycosylation of the 47,000 and the 54,000 proteins was studied by labelling control and tunicamycin-treated cells with $1,6\text{-}^3\text{H-N-acetylglucosamine}$ (15 Ci mmol^{-1}) and $2\text{-}^3\text{H-mannose}$ (15 Ci mmol^{-1}) ($30 \mu\text{Ci ml}^{-1}$ each) for 10 min at 4 h after infection with SFV. The cultures were solubilised in 2% SDS and equal samples (approximately 0.2×10^6 cells) of each cell preparation were run on SDS gels (see Fig. 1). Gels were cut into 2-mm pieces and viral proteins were eluted and measured for radioactivity as described previously¹⁵. The control cells contained 2,100 c.p.m. $^3\text{H-mannose}$ label in p62 and 500 c.p.m. in E1. The corresponding $^3\text{H-N-acetylglucosamine}$ radioactivity found in p62 and E1 was 420 c.p.m.

and 90 c.p.m., respectively. Less than 5% of the radioactive carbohydrate was incorporated into either the 47,000 or the 54,000 proteins compared with controls. This confirms the earlier finding of Schwarz *et al.*¹⁶ and Leavitt *et al.*¹⁷ that these two proteins are non-glycosylated (see also refs 18 and 19).

We also tested the binding of the 47,000 and the 54,000 proteins to concanavalin-A-Sepharose columns. About 5% of the 47,000 and the 54,000 proteins was bound to the column in conditions (0.05 M Tris, pH 7.4, 0.15 M NaCl, 0.5% Triton X-100) in which about 90% of the p62 and E1 was bound to the column (not shown). This provides additional evidence for the lack of mannose residues in the 47,000 and the 54,000 proteins.

The identification of the 47,000 and the 54,000 proteins was achieved by comparing the 2-nitro-5-thiocyanobenzoic acid (NTCB) peptides derived from these proteins with those of the E1 and the p62 glycoproteins. Figure 2*a-d* shows that the 47,000 protein contains the E1 sequence of amino acids and suggests that the 54,000 protein contains the p62 sequence. Both the NH_2 -terminal peptide of E1 and the peptide characterising its COOH-terminal end³⁴ were found in the 47,000 protein (arrows in Fig. 2*a*). The presence of an intact NH_2 -terminal peptide of E1 in the 47,000 species demonstrates unequivocally that correct p62-E1 cleavage is taking place in conditions in which glycosylation is inhibited. Larger NTCB

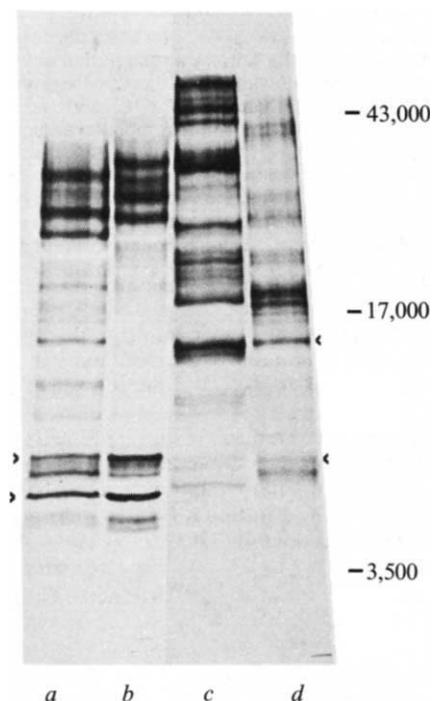


Fig. 2 Identification of the 47,000 and the 54,000 proteins produced in SFV-infected cells in the presence of tunicamycin. ^{35}S -labelled membrane proteins of microsomes ($50 \mu\text{l}$) from control and tunicamycin-treated cells were separated by electrophoresis on 11% SDS-acrylamide gels according to Neville³¹. The 47,000 and the 54,000 proteins, and the E1 and the p62 proteins, were eluted, lyophilised and cleaved at their cysteine residues with NTCB as described³⁴. The resulting NTCB peptides were analysed by electrophoresis on a 15–22% gradient gel. Labelled peptides were visualised by fluorography³⁰. *a*, NTCB peptides of 47,000 protein; *b*, NTCB peptides of E1 protein; *c*, NTCB peptides of p62 protein; *d*, NTCB peptides of 54,000 protein. Upper arrow in (*a*) indicates a peptide characterising the COOH terminus of E1 and the lower arrow in (*a*) indicates the NH_2 -terminal peptide of E1³³. Arrows in (*d*) indicate comigrating peptides of p62 and the 54,000 protein. The migrations of ovalbumin (43,000), myoglobin (17,200) and the β -chain of insulin (3,500) are indicated on the right side.

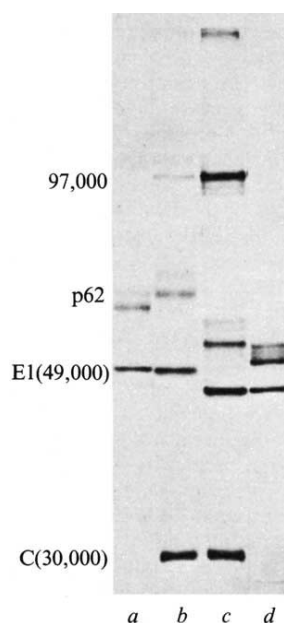


Fig. 3 Viral proteins present in microsomes of control and tunicamycin-treated cells. Effect of protease treatment. Cells that had been infected with SFV in the presence or absence of tunicamycin were pulsed with ^{35}S -methionine as described in Fig. 1. Cells were put on ice and then collected, swelled in hypotonic buffer and broken by homogenisation as described elsewhere³⁴. Rough microsomes with newly synthesised ^{35}S -methionine-labelled SFV proteins were finally isolated by density gradient centrifugation²⁰. Samples of the microsome preparations were analysed, directly or after protease treatment, by 10% SDS gel electrophoresis²⁹. ^{35}S -labelled proteins were visualised by fluorography³⁰. Protease treatment was done by incubating $10\ \mu\text{l}$ microsomes, containing approximately 4×10^4 c.p.m. ^{35}S -methionine and $50\ \mu\text{g}$ protein, with $75\ \mu\text{g}$ proteinase K in $40\ \mu\text{l}$ $0.05\ \text{M}$ Tris, $\text{pH}\ 7.4$, $0.1\ \text{M}$ NaCl for $15\ \text{min}$ at 37°C . After incubation, $50\ \mu\text{g}$ of the protease inhibitor PMSF was added, and the remaining proteins precipitated in 10% TCA and electrophoresed²⁹. About 50% of the amount of the membrane proteins was lost during protease treatment. This is most likely due to unsealed microsomal vesicles present in the preparation³². For analyses of proteins of untreated microsomes $5\ \mu\text{l}$ -volumes of these were precipitated in TCA and electrophoresed. Approximate molecular weights of new protein bands were calculated from their positions relative to those of the known proteins. Densitometric tracing of protein bands shows that 40% of the viral proteins (estimated as ^{35}S -methionine radioactivity) present in control microsomes is C protein, 27% E1, 25% p62 and 12% 97,000 protein. In microsomes of tunicamycin-treated cells, 26% of the viral proteins present is C protein, 23% E1, 26% p62 and 25% 97,000 protein. *a*, Protease-treated control microsomes; *b*, control microsomes; *c*, microsomes of tunicamycin-treated cells; *d*, protease-treated microsomes of tunicamycin-treated cells.

peptides of the 47,000 protein are migrating faster than those of the control. This is expected because these normally contain carbohydrate (H. G., unpublished). As all major NTCB peptides of p62 are glycosylated, it is difficult to find correlations with the non-glycosylated NTCB peptides of the 54,000 protein. Some comigrating peptides, however, can be seen (arrows in Fig. 3d). The fact that correct p62-E1 cleavage occurs, the similar double-banded appearance of both the p62 and the 54,000 protein and the membrane location of the 54,000 protein (described below), leave little doubt that the 54,000 protein indeed contains the p62 amino acid sequence.

To determine whether the non-glycosylated forms of the viral membrane proteins become correctly inserted into the lipid membrane during synthesis, we isolated rough microsomes, containing newly synthesised and ^{35}S -methionine-

labelled viral proteins, from tunicamycin-treated and control cells (Fig. 3). Almost all of the viral proteins made during the pulse, except for about 50% of the C protein, were found in the rough microsomal fraction from both cell preparations. The location of the SFV proteins in the microsome preparations was studied by protease treatment. Only proteins located on the internal side of the microsomal membrane remained protected from protease digestion.

Figure 3a shows the effect of protease treatment on control microsomes. As demonstrated previously²⁰, all of the E1 polypeptide is protected and only the transmembrane 3,000 segment from p62 is removed^{20,34}. The rest of p62 could be seen as a faster migrating double band, with molecular weight about 59,000. The capsid and the 97,000 protein were completely degraded. When microsomes from tunicamycin-treated cells were treated with protease (Fig. 3d), the 47,000 protein (that is, the non-glycosylated E1 protein) remained completely protected, whereas the 54,000 protein (that is, the non-glycosylated p62 protein) was reduced by about 3,000 to an approximate molecular weight of 51,000, seen in Fig. 3d as a double band. The capsid and 97,000 protein were digested. These results show that the location of the SFV proteins in tunicamycin-treated cells is completely analogous to that of the proteins in control cells. We conclude, therefore, that correct insertion of SFV membrane proteins into rough microsomes as well as the correct p62-E1 cleavage occur despite the fact that these proteins are synthesised in conditions of inhibition of glycosylation. Membrane protein glycosylation is thus not necessary for either of these processes.

In infected cells the membrane proteins of SFV are transported from their site of synthesis and initial glycosylation in the rough microsomes to the plasma membrane²¹. From here they are specifically incorporated by a budding process²² into mature virions probably through direct nucleocapsid-membrane glycoprotein interactions²³. The exact intracellular pathway for the SFV membrane proteins as well as the segregating forces that bring about the movement of the membrane proteins are not known. It has been suggested that the glycosylation of membrane proteins is important for these processes. Interestingly enough, the non-glycosylated SFV membrane glycoproteins in tunicamycin-treated cells cannot be detected on the plasma membrane¹⁷. It is possible that these membrane proteins are unable to leave their site of synthesis in the rough microsomes, as has been suggested for the IgA and IgE proteins produced by MOPC315 mice plasma cells and IR162 rat plasma cells in the presence of tunicamycin (ref. 24; see also refs 25-27). Correctly inserted and proteolytically processed membrane ectoproteins that are devoid of carbohydrate will certainly prove to be valuable controls in further studies in this field.

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Experimental acceleration and slowing of REM sleep ultradian rhythm by cholinergic agonist and antagonist

MAMMALIAN sleep is characterised by the cyclical occurrence of rapid eye movement (REM) and non-REM sleep. In humans, REM sleep (consisting of a desynchronised electroencephalogram (EEG), rapid eye movements, atonic electromyogram (EMG), and usually accompanied by dreaming), occurs every 90-110 min¹ and is presumed to be under the control of an underlying ultradian oscillatory mechanism². The biological basis of the above ultradian rhythm is unknown. Although in humans the amount of REM sleep itself can be experimentally increased³⁻⁶ or decreased⁷⁻⁹ by pharmacological and physiological means, none of these manipulations has

been shown to alter the frequency of its presumed oscillator. We demonstrate here that a normal 104-min REM sleep ultradian rhythm can be experimentally shortened to a 56-min rhythm by repeated infusions of arecholine, a cholinergic muscarinic agonist¹⁰. Conversely, scopolamine, a cholinergic muscarinic antagonist¹¹, prolonged the interval between successive REM periods. We speculate that acetylcholine may have a specific role in the timing of the ultradian oscillatory mechanism controlling REM sleep.

Seven normal volunteers (four males, three females; mean age 25 yr, range 21-30) were studied on three non-consecutive nights (one 'adaptation' and two experimental nights) in a placebo-controlled, crossover design. Techniques of polygraphic sleep recordings and intravenous infusion of drugs during sleep were as described previously¹². The drug or placebo treatments administered in random sequence on the two experimental nights were as follows. Some volunteers received first pretreatment with an intramuscular injection of methscopolamine 0.5 mg before retiring (about 23.30) followed by three intravenous infusions of arecholine (infusions I, II and III) each administered over a 2-3-min period. Infusion I (1.5 mg dose) was given 35 min after sleep onset, infusion II (1.0 mg) 30 min after the end of the first REM period, and infusion III (1.0 mg) 30 min after the end of the second REM period. Thus the three infusions were given during the first, second and third non-REM periods, respectively. Other volunteers received first pretreatment with methscopolamine 0.5 mg intramuscularly followed by three placebo (isotonic saline) intravenous infusions. Subjects were run in pairs or groups of three on each night so that the timing of the placebo infusions was 'yoked' inasmuch as it was possible to the time of arecholine infusions given to another subject (undergoing drug treatment condition). Occasionally, if a subject was found to be in REM sleep when it was time for a placebo infusion, we waited for the next non-REM period to give the infusion. Methscopolamine is a peripheral anticholinergic agent given to counteract the peripheral side-effects of arecholine. By itself it has been reported to have no effects on human EEG sleep⁹.

Table 1 Effect of arecholine and scopolamine on REM sleep

	Experiment I			Experiment II		
	Placebo	Arecholine	Paired <i>t</i> -test (two-tailed)	Placebo	Scopolamine	Paired <i>t</i> -test (two-tailed)
(1) REM ₁ -REM ₂ (min)	103.4±2.6	51.1±2.3	<i>P</i> <0.01	98.6±7.9	205.4±11.8	<i>P</i> <0.01
(2) REM ₂ -REM ₃ (min)	104.1±5.9	59.8±8.5	<i>P</i> <0.01	103.2±8.4	151.1±9.2 (<i>n</i> = 5)	<i>P</i> <0.05 (5 matched pairs)
(3) Mean of (1) and (2) (min)	103.8±6	55.5±7.1	<i>P</i> <0.01	100.9±8.1	175.6±11.1	<i>P</i> <0.05 (5 matched pairs)
(4) REM ₃ -REM ₄ (min)	90.6±11.1 (<i>n</i> = 4)	106.1±8.9	NS	94.4±6.6 (<i>n</i> = 5)	—	
(5) REM ₄ -REM ₅ (min)	—	90.5±7.5 (<i>n</i> = 6)		—	—	
(6) Percentage REM time	20.1±1.2	24.8±1.9	<i>P</i> <0.01	22.2±1.9	9.7±1.8	<i>P</i> <0.01
(7) No. of REM periods	3.71±0.29	5±0.22	<i>P</i> <0.01	4±0.36	2.9±0.4	<i>P</i> <0.01

REM-REM refers to the interval between the onset of one REM period to the beginning of the next. Sleep cycles were analysed according to criteria derived from Feinberg¹⁷. According to these criteria, a REM period was accepted as complete only if it was at least 5 min in duration. REM periods interrupted by non-REM sleep of 25 min or less were treated as single periods; if more than 25 min of non-REM sleep intervened they were scored as two separate REM periods. The final REM period of the night was accepted as complete only if followed by 5 min of non-REM sleep. The final non-REM period was accepted as complete only if followed by 5 min of REM sleep. Awake time, both across the night and during individual REM and non-REM periods was not significantly different between drug and placebo conditions in both experiments and was therefore not subtracted when calculating REM-REM intervals. Other sleep parameters during experiment I were as follows. On placebo, REM density (an index of eye movement intensity scored on a scale of 0-8) was = 1.6±0.2 units (mean±s.e.m.), total sleep time = 421.3±10.4 min, awake time = 14.6±5 min, delta sleep (stages 3 and 4) percentage = 15±2.5%. On arecholine nights, REM density = 1.4±0.3 units, total sleep time = 416.9±19.2 min, awake time = 18.1±8 min, delta percentage = 13.7±3.2%. During experiment II, the data were as follows. On placebo, total sleep time = 431.8±16 min, REM density = 1.9±0.2 units, awake time = 16.7±3.1 min, and delta sleep percentage = 13.5±2.5%. After scopolamine, total sleep time = 402.6±16.1 min, REM density = 1.3±0.1 units, awake time = 20.1±5.5 min, and delta sleep percentage = 12.9±3.3%. Values are means±s.e.m.

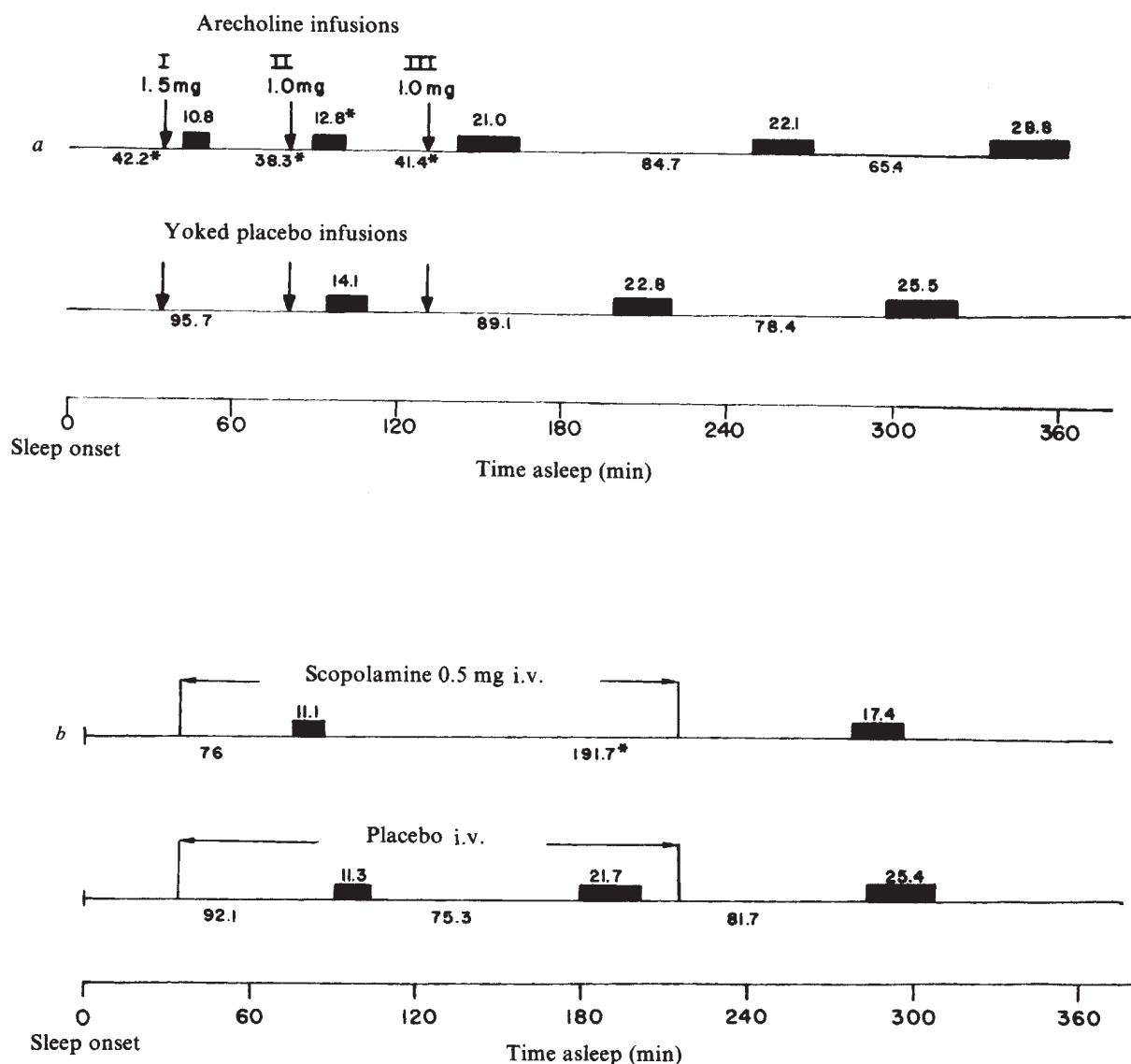


Fig. 1 *a*, Experiment I: shortening of REM sleep ultradian rhythm by arecholine infusions ($n=7$). *b*, Experiment II: lengthening of REM-REM interval by intravenous infusion of scopolamine over a period of 2 h ($n=7$). * $P<0.01$. Bars represent REM time. During experiment II, five out of the seven subjects had an intact third non-REM period after scopolamine infusion. The mean $\pm$ s.e.m. of the third non-REM period after scopolamine (130.7 ± 12.3 min) was significantly longer than the mean of the five matched placebo non-REM periods (79.8 ± 10 min, $P<0.05$ two-tailed paired t test).

All infusions were administered by a single investigator based on his judgment regarding sleep onset (first spindle or k complex) and the end of the first and second REM periods. No markings were made on the polygraphic record so as not to bias the judgment of a second investigator who scored the records. All records were scored without knowledge of the drug status of subjects according to standard criteria¹³.

As shown in Fig. 1 (experiment I), each of the three infusions of arecholine induced the onset of the first, second and third REM period, respectively. The durations of the first, second and third non-REM periods were significantly reduced by arecholine compared with placebo. As indicated in Table 1, arecholine significantly shortened the interval between the first and second (REM₁-REM₂) and the second and third REM periods (REM₂-REM₃), resulting in a mean REM cycle period length of 55.5 min compared with the length on placebo of 103.8 min. The REM-REM intervals obtained on placebo in our study are well within the range observed by others¹⁴. The REM₃-REM₄ and REM₄-REM₅ ($n=6$) intervals on the arecholine night were within the normal range, indicating that the

action of arecholine was shortlived and did not extend into the fourth non-REM period.

The number of REM periods, and the total REM time and percentage, were significantly higher on arecholine nights (Table 1). The duration of the first and third REM periods tended to be shorter but not significantly; the second REM period was, however, significantly shorter after arecholine compared with placebo (Fig. 1). Other sleep parameters, such as REM density, total sleep time, awake time, and percentage of delta sleep (stages 3 and 4) were unchanged (Table 1).

In a second experiment (II) we investigated whether a cholinergic antagonist would produce an opposite effect (namely, prolongation of REM-REM interval) to that of arecholine. We administered scopolamine to a separate group of seven normal volunteers (six male, one female; mean age 23.7 yr, range 20-27). After a night of adaptation, subjects received a 180-min intravenous infusion beginning at 35 min and ending at 215 min after sleep onset, of either scopolamine (0.5 mg at the rate of 0.003 mg min⁻¹) or placebo (isotonic saline at the rate of 0.5 ml min⁻¹) in random order.

As shown in Fig. 1 (experiment II), the latency to the onset of the first REM period was not different on scopolamine and placebo nights. This is not surprising as the dose of scopolamine administered up to the time of onset of the first REM period was probably too low to exert significant central anticholinergic effects. The second and the third non-REM periods (see Fig. 1) were, however, significantly prolonged by scopolamine compared with placebo. Similarly, the REM₁-REM₂ and the REM₂-REM₃ intervals were also significantly lengthened after scopolamine (Table 1). The total REM time and percentage were significantly reduced and this was due to the decreased number of REM periods present during the night, as the duration of individual REM periods remained unchanged. Other sleep parameters were not affected by scopolamine (Table 1).

In two earlier studies^{12,15} we reported that a single infusion of either physostigmine 0.5 mg or arecholine 1.0 mg and 1.5 mg, given 35 min after sleep onset, consistently induced the onset of the first REM period. Once the first REM period was moved forward, the lengths of the subsequent REM-non-REM cycles were not different from placebo. In the present study, however, the timed multiple infusions of arecholine shortened not only the latency to onset of the first REM period, but also the REM₁-REM₂ and the REM₂-REM₃ intervals. Conversely, scopolamine prolonged the REM₁-REM₂ and REM₂-REM₃ intervals. A rhythmic function such as REM rhythm can be altered either by affecting the endogenous (free-running) rhythm or by a synchroniser. As arecholine was given in timed pulses we cannot be sure if its effects were on the frequency of the endogenous rhythm or if it reset the phase. On the other hand, the results with scopolamine can probably be attributed to a direct effect on the oscillator frequency, as it was given by slow continuous infusion. Our data are consistent with the model proposed by Hobson and others¹⁶ of a reciprocal interaction of an excitatory, presumably cholinergic neuronal group (pontine gigantocellular tegmental field) and an inhibitory presumably noradrenergic neuronal group in the locus coeruleus as a physiological basis of sleep cycle oscillation.

It is also of interest that the modest increase in the percentage of REM time from 20.1% to 24.8% in this study and from 18.1% to 24.7% after administration of a single dose of arecholine 1.5 mg in the earlier study¹⁵, resulted mainly from an increase in the number and not duration of individual REM periods. In fact, the second arecholine-induced REM period in the present study was significantly shorter than the matched placebo REM period. These data suggest that only the timing mechanism of REM sleep is under the excitatory control of brain acetylcholine and is dissociable from factors determining the duration of REM periods; and that there may be a limit or 'ceiling' after which total REM time cannot be increased by arecholine, as both the single infusion of 1.5 mg and multiple infusions (totalling 3.5 mg) produce the same increase in percentage of REM sleep.

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Direct biochemical demonstration of two types of α -adrenoreceptor in rat brain

IN the peripheral sympathetic nervous system the transmission of impulses from postganglionic sympathetic neurones to their effector cells is determined by receptors both on the effector cell ('postsynaptic') and on the postganglionic nerve endings ('presynaptic'). The presynaptic receptors seem to be involved in the regulation of noradrenaline release during nerve stimulation^{1,2}. The limited pharmacological data available suggest that in the central nervous system there is more than one class of α -adrenergic receptors and that they are involved in the regulation of central adrenergic transmission³. We describe here the direct biochemical identification of two classes of α adrenoreceptor in rat brain membranes.

α -Adrenoreceptors in the central nervous system have been specifically identified and characterised in several laboratories^{4,5}. Using the potent α -adrenoreceptor antagonist, ³H-dihydroergocryptine (³H-DHE), we have confirmed in preliminary experiments that it binds to sites highly suggestive of α -adrenoreceptors in rat brain membranes⁶. Brain membranes were prepared by a modification of the method of Greenberg *et al.*⁷. In brief, male Wistar rats (approximate weight 300 g) were killed by decapitation and their brains were removed rapidly and placed in ice-cold 0.9% NaCl. Cerebella were removed and the remaining brain was homogenised in 30-40 volumes (w/v) of ice-cold buffer (0.25 $\times 10^{-3}$ M sucrose, 1 $\times 10^{-3}$ M MgCl₂, 5 $\times 10^{-3}$ M Tris-HCl, 0.05% ascorbic acid, pH 7.4) for two 10-s periods using a TP Ultra Turrax homogeniser at maximum speed. The homogenate was centrifuged at 49,000g for 10 min at 4°C in a Sorvall RC 2B centrifuge. The pellet was resuspended in fresh ice-cold buffer, rehomogenised and centrifuged as previously and then resuspended in ice-cold incubation buffer (5 $\times 10^{-2}$ M Tris-HCl, 1 $\times 10^{-2}$ M MgCl₂, 0.05% ascorbic acid, pH 7.4) for use in the binding assay. ³H-DHE (NEN, specific activity 23.7 Ci mmol⁻¹, 1 $\times 10^{-9}$ M-2 $\times 10^{-8}$ M) and crude membrane suspension were incubated for 20 min at 25°C in a total of 150 μ l of incubation mixture. Protein concentration in the incubation mixture, as measured by the method of Lowry *et al.*⁸ was approximately 2.5 mg ml⁻¹. Incubations were terminated by diluting 100 μ l of the incubation mixture with 2 ml of ice-cold incubation buffer followed by rapid filtration through Whatman GF/C glass fibre filters. Filters were washed rapidly with 20 ml of ice-cold incubation buffer. After drying, filters were counted in 7 ml of Permafluor in a Packard PRIAS PL Tri-carb liquid scintillation counter with an efficiency of 42%. Specific binding of ³H-DHE (defined as total radioactivity bound to membrane protein minus binding which was not displaced by a high concentration (1 $\times 10^{-5}$ M) of the potent α antagonist, phenoxybenzamine) to these membranes was rapid (equilibrium being attained at approximately 18 min at 25°C), reversible, saturable and of high affinity, having an apparent equilibrium dissociation constant (K_d) of 4.7 $\times 10^{-9}$ M, in agreement with previous experience⁵. The order of potency of several adrenergically active agents in competing for these binding sites was comparable to that previously reported⁵ and was: phentolamine (half

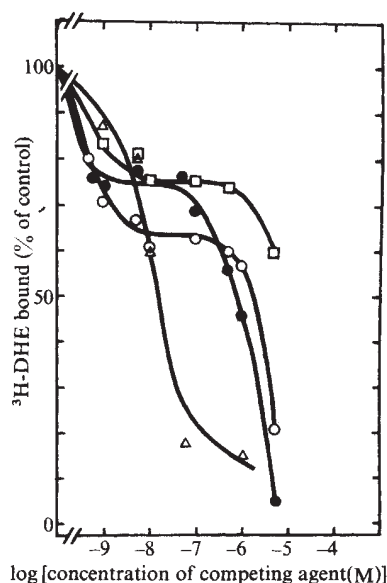


Fig. 1 Curves of displacement of ^3H -DHE (6×10^{-9} M) by increasing concentrations of prazosin (●), yohimbine (○), phentolamine (△) and dopamine (□). The curves represent the percentage bound of total specific binding (that is in the presence of 10^{-5} M phentolamine) of ^3H -DHE to rat brain membranes with increasing concentrations of the drugs. The curves are representative of four experiments performed in duplicate.

maximal inhibition of ^3H -DHE binding, $0.023 \mu\text{M}$) > phenoxybenzamine ($0.099 \mu\text{M}$) > (-)-adrenaline ($0.108 \mu\text{M}$) > clonidine ($0.298 \mu\text{M}$) > (-)-noradrenaline ($0.988 \mu\text{M}$) > (-)-isoprenaline ($19.95 \mu\text{M}$) > ($\pm$)-propranolol ($70.3 \mu\text{M}$) > (-)-dopa ($201 \mu\text{M}$). In all experiments phentolamine seemed to be more than four times as potent a competitor of ^3H -DHE binding as phenoxybenzamine. In the peripheral sympathetic nervous system it seems that phenoxybenzamine may be up to 100 times more effective in blocking postsynaptic than presynaptic α -adrenoreceptor sites but that phentolamine is equipotent in blocking these two sites⁹. Even though this difference was not as marked in our experiments with brain, it indicated that phenoxybenzamine may not be as effective as phentolamine in displacing ^3H -DHE from a 'second' class of α -adrenoreceptor sites. In view of this and of the pharmacological data previously mentioned, we investigated whether ^3H -DHE binds to more than one class of high affinity α -adrenergic site in rat brain membranes.

^3H -DHE-binding to brain membranes was achieved first in the presence of increasing concentrations of prazosin (Pfizer) and then in the presence of increasing concentrations of yohimbine (Sigma), α -adrenoreceptor antagonists acting preferentially on postsynaptic and presynaptic α -adrenoreceptors, respectively, in the peripheral nervous system^{10,11}. Figure 1 shows the displacement characteristics of ^3H -DHE by prazosin and yohimbine. Initially prazosin displaces approximately 35% of specific ^3H -DHE-binding sites in rat brain and this competition is maximal at concentrations between 1×10^{-8} M and 5×10^{-7} M. On the other hand, yohimbine at first displaces approximately 25% of the radioligand and seems to saturate this class of sites at concentrations between 10^{-9} M and 5×10^{-8} M. Thus initially yohimbine plus prazosin displace approximately 60% of specific ^3H -DHE binding sites. In brain ^3H -DHE may also bind to dopaminergic sites¹² and Fig. 1 shows that dopamine displaces approximately 25% of specific ^3H -DHE-binding sites. Possible binding of ^3H -DHE to serotonin receptors⁵, or experimental error, could account for the remaining 15%. With increasing concentrations, each drug competes for ^3H -DHE-binding to membrane protein in a biphasic manner.

The inhibitory effect of prazosin and yohimbine on ^3H -DHE binding reflects the affinity of drug-receptor interactions. At low

concentrations yohimbine and prazosin appear to bind with high affinities to sites which may be different, whereas at high concentrations both drugs seem to have a similar affinity for binding sites. To investigate whether the high-affinity binding sites for yohimbine and prazosin belong to two distinct classes, we examined concentration-dependent binding of ^3H -DHE in the presence of prazosin or yohimbine. Figure 2 demonstrates that ^3H -DHE binds with limited capacity to two classes of sites. Scatchard analysis (insert) demonstrates the affinity of binding of the radioligand to both classes of sites. The K_d values for ^3H -DHE binding to sites in the presence of yohimbine or prazosin were 4.7 ± 1.3 nM (mean $\pm$ s.e.m.) and 3.3 ± 0.6 nM, respectively. The K_d values for these two binding sites were not significantly different by Student's t test ($t = 0.98$). We have called ' α_1 ' the prazosin sites and ' α_2 ' the yohimbine sites, in agreement with a previous suggestion¹³. It should be noted, however, that whole brains (minus cerebella) were used in these studies and that the distribution of these two classes of sites and the affinity of the binding sites for the ligand may differ in different areas of the brain.

In the peripheral sympathetic nervous system pharmacological experiments have made possible the classification of α -adrenoreceptors into 'presynaptic' and 'postsynaptic' types. Pharmacological agents such as yohimbine, piperoxane and clonidine have been shown to have a preferential action on presynaptic receptors, and drugs such as prazosin and phenoxybenzamine to act principally on receptors on the effector cell. We therefore investigate whether the two types of α -adrenoreceptor in the central nervous system of the rat had some similarity with the peripheral receptors. Table 1 shows the effect of saturating each class of receptors with either yohimbine or prazosin and displacing ^3H -DHE from the remaining sites with either clonidine or piperoxane. When yohimbine is in excess, ^3H -DHE binds preferentially to the ' α_1 ' receptor sites (prazosin sites). High concentrations of clonidine or piperoxane have no effect in displacing the radioligand from these sites. Prazosin, however, at 1×10^{-9} M displaces about 35% of ^3H -DHE from these sites. Conversely, when prazosin is in excess, ^3H -DHE binds preferentially to the ' α_2 ' receptor sites

Fig. 2 Saturability and Scatchard analysis of ^3H -DHE-binding sites in rat brain. △, Total binding of ^3H -DHE to rat brain membranes. The other two curves show binding of ^3H -DHE in the presence of a constant concentration of 10^{-8} M prazosin (●, ' α_2 ' sites) or of 10^{-7} M yohimbine (○, ' α_1 ' sites). At these two concentrations, prazosin and yohimbine show maximal inhibition of ^3H -DHE-binding to the two classes of sites on plasma membranes. Scatchard analysis of the top curve shows one class of binding sites with an equilibrium dissociation constant (K_d) of $4.7 \pm 0.13 \times 10^{-9}$ M. Scatchard analysis of the two lower curves shows two classes of binding sites with K_d values as in the text. The Scatchard plots were fitted by linear regression. The saturation curves and Scatchard plots are the means of three experiments performed in duplicate.

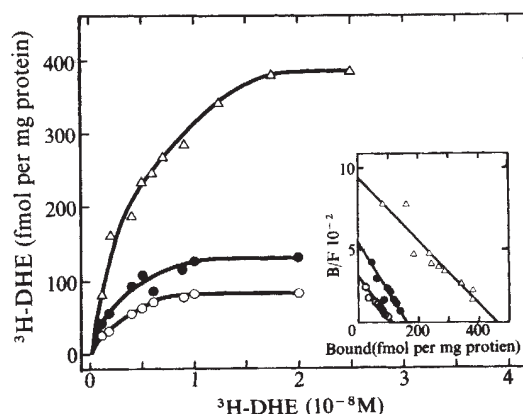


Table 1 Inhibitory effect of clonidine, piperoxane, yohimbine or prazosin on specific ^3H -DHE binding

Incubation mixture	Clonidine (1×10^{-9} M)	Piperoxane (1×10^{-9} M)	Yohimbine (1×10^{-9} M)	Prazosin (1×10^{-9} M)
^3H -DHE (6×10^{-9} M) +	0%	0%	—	35%
Yohimbine (1×10^{-7} M)				
^3H -DHE (6×10^{-9} M) +	12%	28%	20%	—
Prazosin (1×10^{-7} M)				
^3H -DHE (6×10^{-9} M) alone			24%	30%

Plasma membranes were incubated with 6×10^{-9} M ^3H -DHE and either 10^{-7} M yohimbine or 10^{-7} M prazosin in order specifically to saturate the corresponding class of sites in each case. Percentages of total specific c.p.m. displaced from brain membrane protein 30 min after the addition of either 10^{-9} M clonidine or 10^{-9} M piperoxane are shown. Nonspecific binding was defined as binding not displaced by a mixture of 10^{-5} M prazosin and 10^{-5} M yohimbine and specific binding was taken as total counts (that is counts in the presence of yohimbine or prazosin) minus nonspecific counts. The effects of these drugs on the binding of ^3H -DHE alone are also shown. Displacement of ^3H -DHE alone by piperoxane or clonidine was not examined.

(yohimbine sites) and clonidine and piperoxane have an inhibitory effect on the binding. In summary, the ' α_2 ' receptor sites exhibit a preferential affinity for prazosin, whereas ' α_2 ' sites do so for yohimbine and clonidine. Even though these two classes of receptors seem to show specificities for various drugs comparable to the presynaptic and postsynaptic receptors in the peripheral nervous system, pharmacological data are scarce and do not enable us to equate these two central sites with the peripheral presynaptic and postsynaptic receptors. It is possible, however, that these two central sites are similar to the presynaptic and postsynaptic sites described in the periphery. After the demonstration of two classes of α -adrenoreceptors in the brain it may no longer be sufficient to refer to central α -adrenoreceptors as one class of sites.

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Slow relaxations of acetylcholine-induced potassium currents in *Aplysia* neurones

RECENT studies of the vertebrate endplate have shown that similar evaluations of the lifetime of the channels opened by acetylcholine (ACh) can be obtained by four different methods¹: from the decay of the endplate current²; from the ACh noise spectrum^{3,4}; from voltage-jump-induced relaxations^{5,6}; and from single-channel recordings⁷. Similar agreement has been found in the study of other systems⁸⁻¹¹. We now report on exceptionally slow voltage-jump-induced relaxations which we have observed in *Aplysia* neurones during the study of a slow inhibitory ACh response caused by a selective increase in potassium ion permeability¹². We have found that the time constant of the voltage-jump-induced relaxations is larger than that of the decay of the synaptic currents. These relaxations present several peculiar properties, most of which can be explained by a kinetic scheme derived from the Hodgkin-Huxley model of the potassium system in the squid axon¹³.

The experiments were carried out using the 'medial cells' of the pleural ganglion of *Aplysia californica*¹². The seawater composition was (in mM): NaCl 480; KCl 10; MgCl₂ 50; CaCl₂ 10; Tris-HCl 10 (pH 7.8). All experiments were carried out at room temperature (22-27°C) with a two-electrode conventional voltage-clamp⁹. The medial cells have two types of ACh receptors: one triggering a Cl⁻ response, the other a K⁺ response¹². The Cl⁻ response was eliminated in most experiments by the addition of 10^{-3} M tubocurarine. Some experiments were carried out in the absence of tubocurarine, and the Cl⁻ response was then eliminated by taking advantage of the fact that it is not observed if ACh or carbamylcholine (Carb) are applied ionophoretically at some distance from the neurone^{14,15}. The slow relaxations observed in the presence of tubocurarine, and described below, could also be observed in these conditions.

A voltage jump experiment is presented in Fig. 1. A voltage pulse, from -50 to -110 mV, was applied first in the absence of Carb, then during the plateau of the response elicited by bath application of 2×10^{-5} M Carb. The two potential values were equidistant from the reversal potential of the response, which was -80 mV in this experiment (see ref. 12 and Figs 2 and 3). In Fig. 1b, the currents recorded during the two hyperpolarising pulses have been superimposed. Their difference (measured by the interval between the traces labelled A and B) was equal to the Carb-induced current. At the holding potential (-50 mV) the steady-state-induced current I_{ss} (-50) was outward. When the voltage was brought to -110 mV, the Carb-induced current turned inward, as expected from the inversion of the driving force. It increased from an 'instantaneous' value I_{in} (-110) to a steady-state value I_{ss} (-110). This increase was exponential, with a time constant τ (-110) = 2 s (Fig. 1c). When the voltage was stepped back to -50 mV, the Carb-induced current became outward again and now decreased from an instantaneous value,

I_{in} (-50), to the steady-state value I_{ss} (-50). The decrease was again exponential (Fig. 1c) and its time constant was $\tau(-50) = 1.3$ s.

Knowing the reversal potential of the Carb-induced current ($E_K = -80$ mV in this experiment), it is possible to calculate the Carb-induced conductance, g_{Carb} , from

$$g_{Carb} = I_{Carb} / (V - E_K) \quad (1)$$

where I_{Carb} is the Carb-induced current, and where the driving force ($V - E_K$) takes alternatively the values of +30 and -30 mV. The resulting plot (Fig. 1d) shows that, after the hyperpolarising pulse, g_{Carb} first instantaneously decreased, and then slowly increased. An inverse evolution was observed on depolarisation.

Instantaneous variations of g can be readily measured in Fig. 1b by the ratios $I_{in}(-110)/I_{ss}(-50)$ and $I_{ss}(-110)/I_{in}(-50)$ at the onset and at the end of the hyperpolarising pulse, respectively. The two ratios are nearly identical: their experimental values are 0.31 and 0.29. We interpret this 'instantaneous rectification' as reflecting the $I-V$ curve of one open channel. It is likely to correspond to the observations by Ginsborg and Kado¹⁵ in the same neurones. As noted by these authors, its 'outward' direction is that predicted from the Goldman equation.

The time-dependent evolution of g_{Carb} would by itself lead to an inward-going rectification which in the stationary state tends to cancel the instantaneous outward-going rectification. It is probable that the time-dependent change of conductance is due to a change in the number N of open channels, as demonstrated in other ACh systems^{5,6,8,9}. N seems to vary by a factor 3.5 both at -110 and at -50 mV in the experiment of Fig. 1. Even steeper variations with voltage (five to sixfold from -40 to -100 mV) were observed at lower Carb concentrations (for example 5×10^{-6} M).

In a further series of voltage-jump experiments, the holding potential was kept constant while the potential reached during

the hyperpolarising pulse was varied (Fig. 2). The time constant of the relaxation observed on repolarisation was independent of the size of the voltage steps, and only dependent on the final potential, as has been found in other preparations^{5,8,9}. The combined results of these experiments indicate that the increase of τ with hyperpolarisation is similar to that observed for excitatory effects in other systems⁴⁻⁹. For example, we found

$$\frac{\tau(-60)}{\tau(-40)} = 1.12 \pm 0.08 \text{ (mean } \pm \text{ s.e., 8 cells)}$$

$$\frac{\tau(-100)}{\tau(-40)} = 1.50 \pm 0.12 \text{ (mean } \pm \text{ s.e., 6 cells)}$$

Experiments of the type illustrated in Fig. 2 were also used to analyse the effect of changes in membrane potential on I_{in} , on I_{ss} and on the steady-state number of open channels, N_{ss} (Fig. 2d-f). With hyperpolarisation, N_{ss} increased more steeply than τ (see also Fig. 1), in contrast with previous findings on other ACh responses^{4,5,8,9}.

The relaxations observed in the presence of ACh were similar to those observed with Carb. For example, at -40 mV, the mean value of $\tau(-40)_{ACh}$ was 2.0 ± 0.5 s, compared with 2.1 ± 0.4 s for $\tau(-40)_{Carb}$. The similarity of the values of τ_{Carb} and τ_{ACh} contrasts with the difference in these values at the frog neuromuscular junction³ or on *Aplysia* neurones excited by ACh⁹.

We then analysed the postsynaptic currents produced by stimulation of the presynaptic neurone described by Kehoe¹⁶ and assumed to be cholinergic in view of the similarity between the effects of its stimulation and those of ionophoretically applied ACh^{12,14,16}. As indicated in Fig. 3, both the increase time and the decay of these synaptic currents are remarkably slow. Both seem to be voltage dependent, and both increase with hyperpolarisation. The time constant τ_{syn} of the decay was measured with varying trains of presynaptic spikes, and at

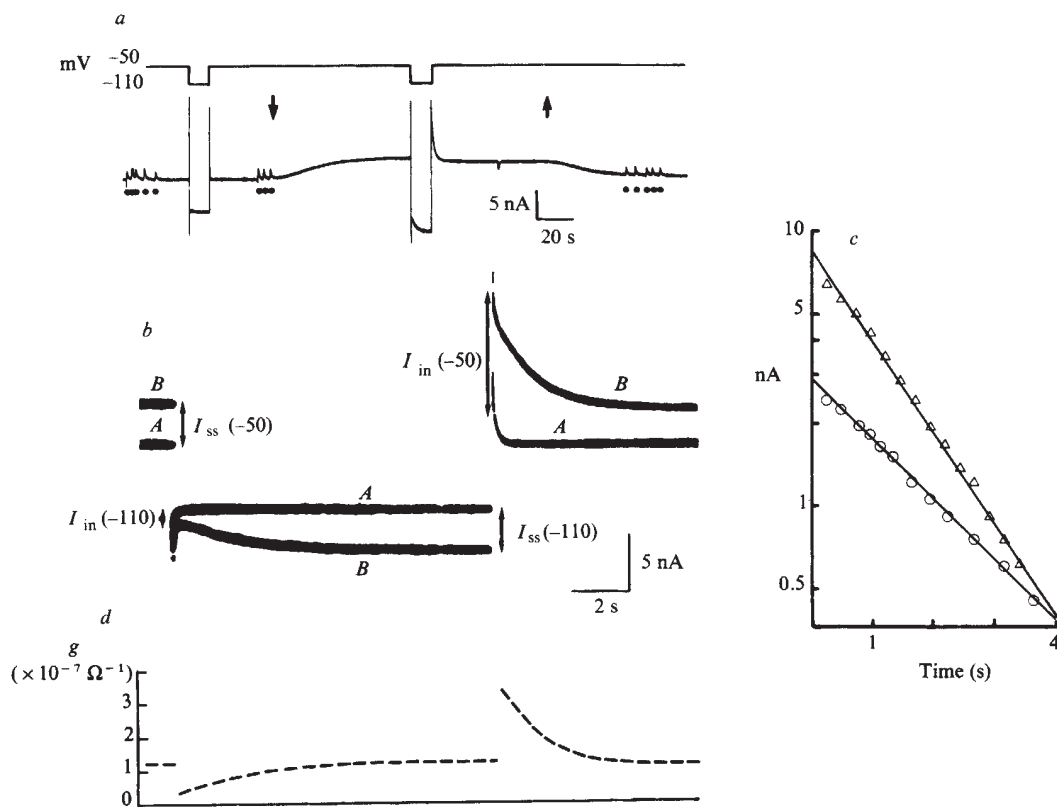
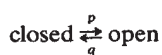


Fig. 1 A relaxation experiment. *a*, Pen record of membrane current during a relaxation experiment. Holding potential -50 mV. An 11-s voltage pulse was applied first in control conditions, then in the presence of 2.5×10^{-5} M Carb (applied between arrows). Outward currents are upward, as in the other figures. Outward deflections (dots) are spontaneous synaptic currents (the downward deflection during the plateau of the response is a perfusion artefact). *b*, Superimposed records of the current in the control (A) and in the presence of Carb (B). The Carb-induced current is the difference between the two traces. It increases with time at -110 mV and decreases at -50 mV. Values of the instantaneous current, $I_{in}(-50)$ and $I_{in}(-110)$, and of the steady-state current, $I_{ss}(-50)$ and $I_{ss}(-110)$, are indicated. *c*, Semi-logarithmic plot of the two relaxations. Both seem to be exponential. Δ , $\tau(-50) = 1.3$ s; $\circ$, $\tau(-110) = 2.0$ s. *d*, Plot of the Carb-induced conductance, g , calculated from *b* and equation (1). g shows instantaneous changes at the onset and at the end of the hyperpolarising pulse, followed by slow variations in the opposite direction. Time scale as in *b*. Temperature 24 °C.

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various membrane potentials. It increased slightly with the duration of the presynaptic train. However, in all cases it was markedly shorter than that of the relaxations measured at the same potential. At -40 mV, the mean value was 0.73 ± 0.22 s.

The voltage dependence of the inhibitory K^+ response to ACh resembles that of the excitatory ACh responses described in other systems^{4,5,8,9}: the time constants of both the voltage-jump-induced relaxation and of the synaptic currents decay increase with hyperpolarisation. However, the time constants of the two processes, measured at the same potential, are different. This cannot be due to diffusion delays at the synapse, or to inadequate control of the potential across the postsynaptic membrane, for such factors would tend to lengthen the decay of the synaptic currents, so that the discrepancy between the two time constants would be even larger than the observed factor of 3. Such a difference is not expected from the kinetic models used to account for ACh excitatory effects. These models all assume two states of the ionic channels, interconverting with constant transition rates, p and q :



(2)

p increases with the agonist concentration; q (and possibly p)

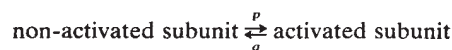
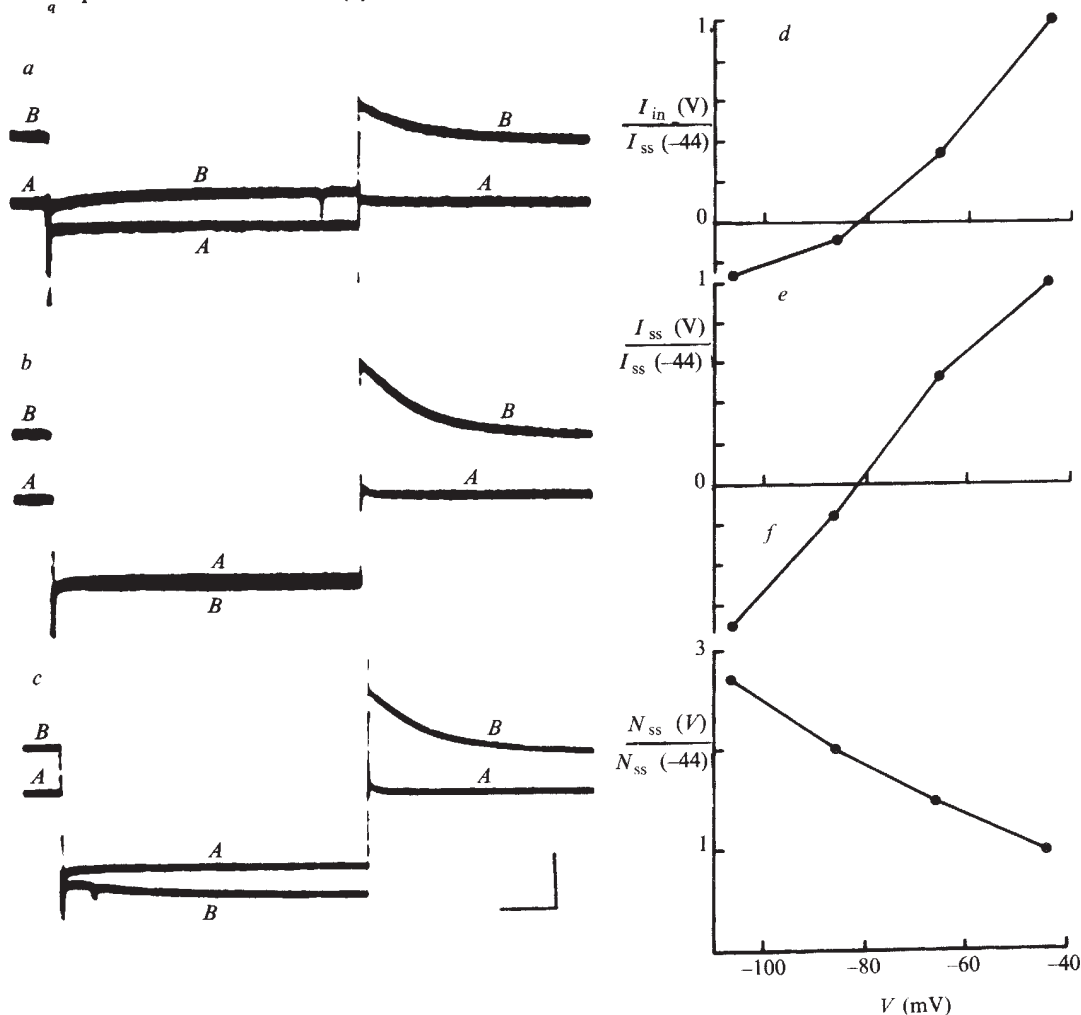


Fig. 2 Instantaneous and steady-state currents. *a*, *b*, and *c*, Three relaxation experiments were carried out on the same cell at 26.5°C , using the same ionophoretically applied dose of Carb. The initial (holding) potential was -44 mV. The amplitude of the hyperpolarising pulse was increased from *a* to *c*: in *a* the potential was brought to -66 mV; in *b* to -86 mV; in *c* to -106 mV. In *a* the driving force was simply reduced during the test pulse; in *b* and in *c* it was inverted. Traces *A* and *B* were obtained in the absence and in the presence of Carb, respectively. The time constants of the 'on' relaxation are 2.2 s in *a*, 3.35 s in *c*. The time constant of the 'off' relaxation is close to 2.0 s in *a*, *b* and *c*. Calibrations: 2 s (*a*, *b*, *c*); 5 nA (*a*, *b*); 10 nA (*c*). *d*, The ratio $I_{in}(V)/I_{ss}(-44)$ (see Fig. 1*b* for the notations) was plotted as a function of the voltage V of the hyperpolarising pulse in *a*, *b* and *c*. As the total number of open



channels is presumably the same before and just after the onset of the hyperpolarising pulse, this ratio should equal the ratio of the elementary currents $i_{el}(V)/i_{el}(-44)$. Thus, the curve reflects the dependence of the elementary current on voltage, normalised to its -44 mV value (therefore the value of 1 at -44 mV). It shows an outward going rectification. *e*, the ratio $I_{ss}(V)/I_{ss}(-44)$ was plotted as a function of V in *a*, *b* and *c*. In this experiment the resulting curve is almost linear, indicating that the time dependent change in conductance tends to cancel the instantaneous outward rectification. *f*, The relative number of open channels, $N_{ss}(V)/N_{ss}(-44)$ in the steady state can be calculated by assuming that the steady-state current, I_{ss} , is the product of the steady-state number of open channels, N_{ss} , and the elementary current flowing through one channel, i_{el} ; and that after a jump the instantaneous current, I_{in} , reflects only a change in i_{el} . In the experiment shown in *a*, *b*, and *c* at the holding potential (-44 mV), $I_{ss}(-44) = N_{ss}(-44) \cdot i_{el}(-44)$. After a jump to a new potential V , $I_{in}(V) = N_{ss}(-44) \cdot i_{el}(V)$ and $I_{ss}(V) = N_{ss}(V) \cdot i_{el}(V)$. Therefore $N_{ss}(V)/N_{ss}(-44) = I_{ss}(V)/I_{in}(V)$. But the value of $N_{ss}(V)/N_{ss}(-44)$ can also be obtained from the analysis of the currents after repolarisation from V to -44 mV. As $I_{in}(-44) = N_{ss}(V) \cdot i_{el}(-44)$ and $I_{ss}(-44) = N_{ss}(-44) \cdot i_{el}(-44)$, one has $N_{ss}(V)/N_{ss}(-44) = I_{in}(-44)/I_{ss}(-44)$. The values plotted in *f* were obtained from this second type of measurement. In this particular experiment the ratio $N_{ss}(V)/N_{ss}(-44)$ varies by a factor e in 65 mV.

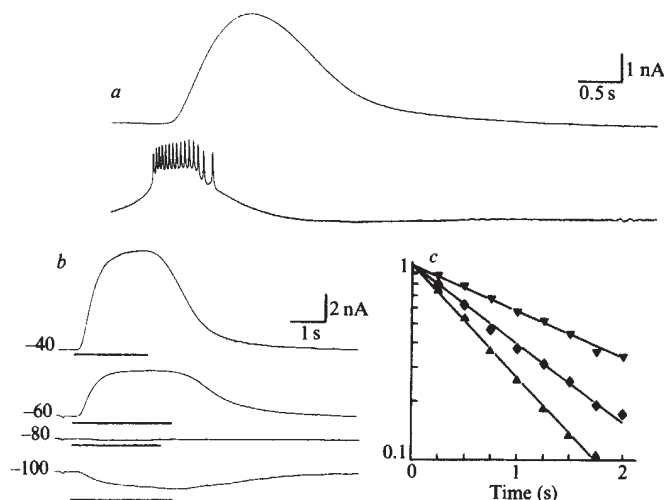


Fig. 3 Time-course of synaptic currents. *a*, Postsynaptic current (upper trace) elicited by stimulation of the presynaptic neurone described in ref. 16 (lower trace). Note the delayed onset of the response, and the lag time (450 ms) between the end of the train of presynaptic spikes and the peak of the postsynaptic current. Membrane potential -50 mV. Time constant of decay 0.65 s. For such short stimulation both the time to peak and the time constant of decay vary with membrane potential (not illustrated). *b*, Responses to somewhat longer presynaptic stimulation (indicated by a bar below each record) at various potentials (from a different cell). The postsynaptic current inverts at -80 mV. *c*, Semi-logarithmic plot of the decays in *b*. The time constants are 0.75 s ($\blacktriangle$), 0.98 s ($\blacklozenge$) and 1.7 s ($\blacktriangledown$) at -40 , -60 and -100 mV, respectively. They are somewhat larger than for a short presynaptic train as in *a*, and they increase with cell hyperpolarisation. The record at -40 mV was obtained with a shorter presynaptic train duration than at -60 and -100 mV; however, the response to a longer train, with a length very similar to those at -60 and -100 mV, had a virtually identical decay time.

depends on membrane potential. The number of open channels (N) will be given by

$$N = N_T n^\alpha$$

where N_T is the total number of channels, and n is a voltage and time-dependent activation parameter similar to that introduced by Hodgkin and Huxley¹³ to describe the kinetics of the K^+ current activation in the squid axon.

Following a sudden perturbation of the system, N will vary following the relation

$$N(t) = N_T [n_\infty + (n_0 - n_\infty) \exp(-t(p + q))]^\alpha \quad (3)$$

where n_0 and n_∞ are the steady-state values of n before and after the perturbation.

If n_0/n_∞ is close to 1, equation 3 is simplified and the relaxation will proceed as a single exponential with a time constant $1/(p + q)$ (which becomes $1/q$ if we further assume that $p \ll q$, that is, that the experiments are made at low agonist concentration). If $n_\infty = 0$, equation 3 also leads to a single exponential, with a time constant $1/aq$.

The first situation is likely to be that encountered after a voltage-jump such as those illustrated in Figs 1 and 2, where N varied by a factor smaller than 4. The second situation ($n_\infty = 0$) is likely to describe that occurring during the decay of the synaptic currents, assuming that the ACh concentration in the cleft decreases 'instantaneously' after the liberation².

In such a scheme the ratio between the time constant of the voltage-jump-induced relaxations and that of the decay of the synaptic currents is α . The value $\alpha = 3$ accounts for our observations.

The model proposed also accounts for additional properties of the relaxations and of the synaptic currents. In particular, it

predicts that the voltage dependence of the steady-state number of open channels (Fig. 2f) is more marked than that of τ_{rel} . It can also explain why the synaptic currents present a slow and sigmoid rise (ref. 20 and Fig. 3) which is strongly dependent on temperature (see Fig. 12 in ref. 21).

The model may have to be refined to account for the long delay between the end of the presynaptic firing and the peak of the postsynaptic current (Fig. 3a) and the fact that the time-course of decay of the synaptic current is not always exponential, and varies somewhat with the duration of the presynaptic train. One possibility to consider is that the activation of the α subunits does not lead instantaneously to the opening of a channel.

The results reported may be relevant in the study of a number of slow synaptic responses, and in particular, the 'muscarinic' responses observed in the vertebrate autonomic system: the slow character of these responses cannot be explained by diffusion delays^{22,23}, and a model resembling the one proposed here has been recently presented²⁴.

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Evidence for extramitochondrial pyruvate dehydrogenase involved in acetylcholine synthesis in nerve endings

THE ultimate step of acetylcholine (ACh) synthesis in cholinergic nerve terminals is the transfer of an acetyl group from one molecule of acetyl coenzyme A (acetyl CoA) to one molecule of choline. This reaction is catalysed by choline acetyltransferase (CAT; EC 2.3.1.6), a cytoplasmic enzyme¹. As revealed by isotopic studies, glucose as well as pyruvate can be used as precursor of the acetyl moiety of ACh in mammalian brain tissue²⁻⁵. From the equal efficiency of ($1\text{-}^{14}\text{C}$) and ($6\text{-}^{14}\text{C}$)glucose in labelling ACh, it was concluded that glucose is

transformed into pyruvate through the Embden-Meyerhof pathway. As (2-¹⁴C) and (3-¹⁴C)pyruvate but not (1-¹⁴C)pyruvate give rise to (¹⁴C)ACh, pyruvate must be decarboxylated by a pyruvate dehydrogenase complex (PDHc; EC 1.2.4.1) to generate acetyl CoA, one of the two substrates of CAT. It is usually assumed that PDHc is exclusively located in mitochondria, and so several attempts have been made to understand how the cytoplasmic CAT is supplied in acetyl CoA. In spite of the great diversity of approaches used, none of the various mechanisms contributing normally to the transfer of acetyl groups through the inner mitochondrial membrane⁶ seem to be involved in the synthesis of ACh in brain cholinergic nerve endings^{3-5,7-10}. An alternative hypothesis was thus proposed which assumed the existence of two populations of PDHc within cholinergic nerve endings. Apart from the ubiquitous intramitochondrial PDHc providing the Krebs cycle with activated acetyl groups, an extramitochondrially located PDHc could exist which would be specifically involved in providing acetyl CoA to the cytoplasmic enzyme, CAT. Acetyl CoA used for the neurotransmitter synthesis would thus be generated in the same subcellular compartment in which ACh is formed¹¹. Experiments are described here which confirm the validity of this hypothesis.

We first compared the respective contributions of (2-¹⁴C)pyruvate and of (³H)pyruvate, deriving from (6-³H)glucose to the synthesis of ACh and to the functioning of the Krebs cycle. Although the synaptosomal preparation used was heterogeneous¹² it was reasonable to assume that ¹⁴CO₂ produced from (2-¹⁴C)pyruvate was representative of the Krebs cycle activity operating in each of the various types of nerve endings present in the preparation. Purified synaptosomes (B fraction) were prepared from rat (Sprague Dawley, Charles River) striata according to the method of Gray and Whittaker¹³. The B band of the discontinuous sucrose gradient was diluted with an equal volume of ice-cold water and was centrifuged at 25,000g for 30 min. The synaptosomal pellet was then resuspended in the following medium (in mM): NaCl, 136; KCl, 5.6; NaHCO₃, 16.2; NaH₂PO₄, 1.2; MgCl₂, 1.2; choline, 0.05; eserine, 0.04. Aliquots (0.18 ml) were incubated at 37°C for 5 min with (2-¹⁴C)pyruvate (23 mCi mmol⁻¹; Commissariat à l'Energie Atomique, France; and/or (6-³H)glucose (0.5 Ci mmol⁻¹; Amersham). To trap the ¹⁴CO₂ formed, a microtube containing a strip of paper impregnated with hyamine (15 µl) was introduced into each propylene tube, which was then closed. The incubations were stopped by injecting through the cap 0.4 ml of a 8% trichloroacetic acid (TCA) solution containing 10 nCi of (³H)choline (10.1 Ci mmol⁻¹; Amersham) as internal recovery standard. Blank values (0 min) were obtained by injecting the TCA solution immediately after the addition of the labelled precursors. Samples were further incubated at 37°C for 30 min to trap all the ¹⁴CO₂ formed. ¹⁴CO₂ was then estimated by placing the hyamine-impregnated paper in scintillation fluid. The (¹⁴C)ACh and (³H)ACh synthesised were first extracted in ethylbutylketone as tetraphenylboron salts, and were then isolated by cellulose thin layer chromatography, using an adaptation of a method previously described¹⁴. The recovery was calculated for each sample by estimating the amount of (³H) radioactivity present in the choline spots.

When (2-¹⁴C)pyruvate was used as the sole source of energy, the rate of (¹⁴C)ACh synthesis increased markedly when the concentration of the labelled precursor was raised from 0.06 to 6 mM (Fig. 1a). The rate of (¹⁴C)ACh synthesis in the presence of 6 mM (2-¹⁴C)pyruvate was close to the *V*_{max}, as the apparent *K*_m of (¹⁴C)ACh formation from (2-¹⁴C)pyruvate in striatal synaptosomes has been previously shown to be 0.6 mM (ref. 10). The (¹⁴C)ACh d.p.m. could be transformed into nmol, as the (2-¹⁴C)pyruvate used for ACh synthesis did not undergo any isotopic dilution¹¹. A similar transformation concerning ¹⁴CO₂ would be irrelevant in view of the isotopic dilution of the labelled precursor occurring in the Krebs cycle¹¹. The Krebs cycle activity, measured by the number of ¹⁴CO₂ d.p.m. pro-

duced, was also stimulated when the (2-¹⁴C)pyruvate concentration was increased from 0.06 mM to 6 mM, though to a lesser extent than the rate of (¹⁴C)ACh synthesis (Fig. 1b).

When (6-³H)glucose (0.44 mM) was substituted for (2-¹⁴C)pyruvate as the sole source of energy, (³H)ACh was synthesised in the isolated nerve endings (Fig. 1a). The (³H)ACh d.p.m. could be transformed into nmol, as it is known that: (1) added labelled glucose molecules used in ACh synthesis do not undergo any isotopic dilution¹⁵; (2) the Embden-Meyerhof pathway leads to the formation of two molecules of pyruvate from one molecule of glucose; (3) pyruvate exchanges one of the three atoms of hydrogen carried by its C3 when converted into the acetyl CoA molecules used for ACh formation; (4) the rate of ACh synthesis from 0.44 mM (6-³H)glucose was found to equal that obtained from 6 mM (2-¹⁴C)pyruvate (Fig. 1a).

When synaptosomes were incubated in the presence of (6-³H)glucose (0.44 mM) and increasing concentrations of (2-¹⁴C)pyruvate (from 0.06 mM to 6 mM), both (³H)ACh and (¹⁴C)ACh were synthesised (Fig. 1a). The total rates of ACh synthesis remained constant and did not differ from those observed with either (6-³H)glucose (0.44 mM) or (2-¹⁴C)pyruvate (6 mM) alone. When the concentration of (2-¹⁴C)pyruvate was increased from 0.06 mM to 6 mM the participation of (6-³H)glucose in the total synthesis of ACh decreased from 92% to 21% (Fig. 1a). These results demonstrated that, with regard to ACh synthesis, externally added (2-¹⁴C)pyruvate is used in preference to (³H)pyruvate produced from (6-³H)glucose within the nerve endings; the substitution was

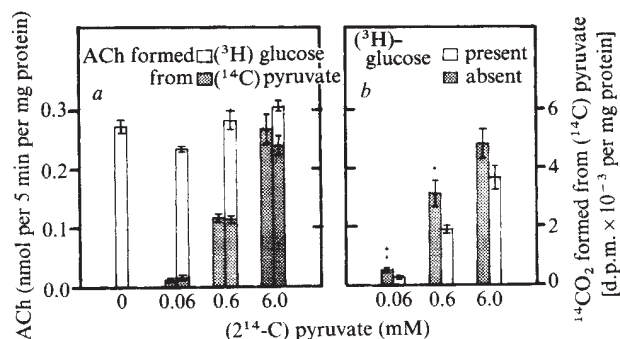


Fig. 1 Formation of ¹⁴CO₂ and of labelled ACh from (2-¹⁴C)pyruvate and (6-³H)glucose in rat striatal synaptosomes. Just before the 5-min incubation at 37°C, 20 µl of either (6-³H)glucose or (2-¹⁴C)pyruvate or a mixture of (6-³H)glucose with various concentrations of (2-¹⁴C)pyruvate, were added to aliquots (0.18 ml) of a synaptosomal suspension prepared from rat striata. The final concentrations were: (6-³H)glucose, 0.44 mM, 42.9 mCi mmol⁻¹; (2-¹⁴C)pyruvate, concentrations as indicated. The specific radioactivity (SRA) was kept constant and was equal to 3.95 mCi mmol⁻¹; protein 4.3 mg ml⁻¹. The nmol of ACh were calculated by correcting the experimentally measured d.p.m. of ACh formed per 5 min per mg protein, by the SRA of the corresponding labelled precursor used. The SRA of (¹⁴C)ACh synthesised was assumed to be equal to that of the added (2-¹⁴C)pyruvate^{11,14}. The SRA of (³H)ACh formed was assumed to be one-third that of the (6-³H)glucose used. Indeed, the added glucose used for ACh synthesis is not isotopically diluted¹⁵; the correction factor is thus of one-half, as one molecule of (6-³H)glucose gives rise to only one molecule of (³H)pyruvate; a further two-thirds correction factor was applied, as it was shown that one of the three atoms of hydrogen carried by C3 of pyruvate is eliminated during ACh formation⁴. Each value is the mean ± s.e.m. of data obtained with groups of five samples. When (6-³H)glucose and (2-¹⁴C)pyruvate were used simultaneously, values were plotted cumulatively (a). The statistical significance of the differences observed was calculated according to the Student's *t*-test¹⁶. When *P* values were higher than 0.05, the differences were not considered to be significant. * *P* = 0.05. ** *P* = 0.01 when compared with samples incubated in the absence of (6-³H)glucose.

almost complete when the external concentration of (2-¹⁴C)-pyruvate reached 6 mM. In contrast, 0.44 mM (6-³H)glucose did not depress the rate of (¹⁴C)ACh synthesis from (2-¹⁴C)pyruvate even when the concentration of the (¹⁴C)precursor was as low as 0.06 mM. The rate of (2-¹⁴C)pyruvate utilisation for (¹⁴C)ACh synthesis was thus independent of the presence or absence of externally added (6-³H)glucose. This situation contrasted with the dependence towards (6-³H)glucose of the rate of utilisation of the molecules of (2-¹⁴C)pyruvate used to feed the Krebs cycle with activated acetyl groups. Indeed, 0.44 mM (6-³H)glucose induced a 45% decrease in the amount of ¹⁴CO₂ produced from 0.06 mM (2-¹⁴C)pyruvate. This percentage inhibition decreased as the concentration of (2-¹⁴C)pyruvate was enhanced: 40% at 0.6 mM, only 25% at 6.0 mM (Fig. 1b).

The simplest interpretation of these results is to assume the existence of two different populations of PDHc within the cholinergic nerve endings (Fig. 2), each of these being fed by two pools of pyruvate. One pool, 'pool ACh', would be involved in the production of acetyl CoA used for the synthesis of ACh, the other, 'pool KC', would be responsible for the formation of acetyl CoA molecules used in the Krebs cycle. With 'pool ACh', (³H)pyruvate produced from (6-³H)glucose was unable to slow down the rate of transformation of the externally added (2-¹⁴C)pyruvate into (¹⁴C)ACh; in contrast, increasing concentrations of (2-¹⁴C)pyruvate markedly depressed the amount of (³H)ACh formed from (6-³H)glucose. The situation concerning the second pool of pyruvate, 'pool KC', was different: competition took place between (³H)acetyl CoA and (¹⁴C)acetyl CoA for entering the Krebs cycle. In fact, (6-³H)glucose reduced the amount of ¹⁴CO₂ formed from (2-¹⁴C)pyruvate, and this inhibition was progressively decreased as the concentration of (2-¹⁴C)pyruvate was raised.

The presence of two pools of PDHc within cholinergic nerve endings was strongly supported by a second experiment. We compared the contribution, to ACh synthesis and to the functioning of the Krebs cycle, of (2-¹⁴C)pyruvate accumulated by passive diffusion in synaptosomes during a 60-min preincubation at 0°C, and of (6-³H)glucose added to this synaptosomal suspension at the onset of incubations carried out at 37°C. As shown by the high amounts of ¹⁴CO₂ recovered at the end of the 5 and 10 min incubations (see Fig. 1 for

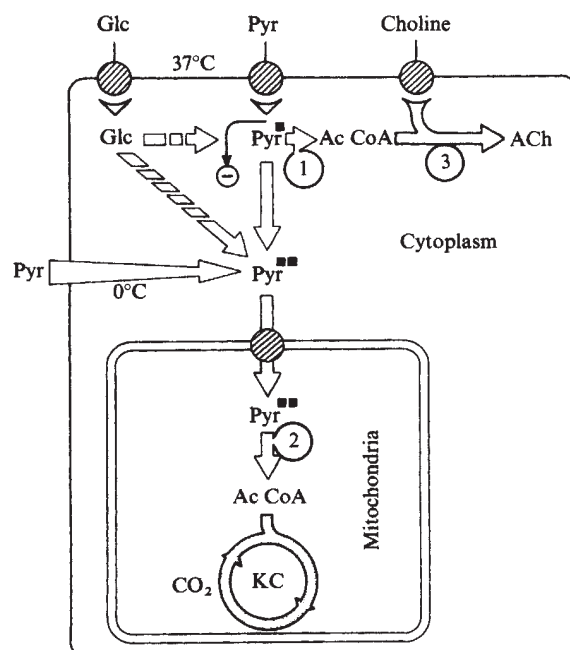


Fig. 2 Schematic representation of the subcellular organisation of ACh synthesis in cholinergic nerve endings. Glc, glucose; Pyr, pyruvate; AcCoA, acetyl coenzyme A; KC, Krebs cycle; 0°C indicates pyruvate penetrating within the synaptosomes during the 60-min preincubation at 0°C. ① and ② represent the reactions catalysed by pyruvate dehydrogenase complexes, ③ the reaction catalysed by choline acetyltransferase; ■ is 'pool ACh' of pyruvate and ■■ 'pool KC' of pyruvate. ⊖ Indicates that (2-¹⁴C)pyruvate penetrating in nerve endings at 37°C is preferentially incorporated in ACh as compared with (³H)pyruvate formed from (6-³H)glucose as shown by the results presented in Table 1. For further explanations see text.

comparison), large amounts of (2-¹⁴C)pyruvate had penetrated into the nerve endings during the preincubation procedure and these molecules readily entered 'pool KC'. As expected, (2-¹⁴C)pyruvate participation in the functioning of the Krebs cycle was reduced when (6-³H)glucose was present during the

Table 1 Synthesis of ¹⁴CO₂ and absence of (¹⁴C)ACh formation in synaptosomes preloaded with (2-¹⁴C)pyruvate

Preincubation (60 min, 0°C)	Saline medium	117 mM (2- ¹⁴ C)pyruvate	117 mM (2- ¹⁴ C)pyruvate
Incubation (37°C)	(6- ³ H)glucose	Saline medium	(6- ³ H)glucose
		¹⁴ CO ₂	¹⁴ CO ₂
0 min		982 ± 89	903 ± 103
5 min		4,204 ± 245	3,500 ± 395
10 min		6,376 ± 709	4,708 ± 81
	(³ H)ACh	(¹⁴ C)ACh	(¹⁴ C)ACh
0 min	350 ± 58	294 ± 18	261 ± 55
5 min	4,205 ± 359	273 ± 29	320 ± 51
10 min	8,260 ± 309	287 ± 35	298 ± 30
			(³ H)ACh
			330 ± 28
			4,461 ± 364
			7,578 ± 358

The B band of synaptosomes diluted with ice-cold water was partitioned in two tubes which were then centrifuged at 25,000g for 30 min. Supernatants were discarded. One pellet was resuspended in 0.3 ml of saline medium, the other pellet in 0.6 ml of the saline medium containing 117 mM of (2-¹⁴C)pyruvate (3.6 mCi mmol⁻¹). This concentration of (2-¹⁴C)pyruvate was chosen to achieve the highest possible intrasynaptosomal level of this compound during preincubation at 0°C. After 60 min the passive diffusion process was stopped by washing the synaptosomes twice with 40 ml ice-cold medium. After the final centrifugation (25,000g; 5 min) synaptosomes were resuspended in the medium and distributed in 0.18-ml aliquots. All the operations so far described were carried out at 0°C. The incubations at 37°C were started after the addition of either 20 µl of the normal saline medium, or 20 µl of a (6-³H)glucose solution. The final concentrations were: when present, (6-³H)glucose, 0.44 mM, 42.1 mCi mmol⁻¹; proteins, 3.0 mg ml⁻¹. The incubations were stopped after 0, 5 and 10 min. ¹⁴CO₂, (³H)ACh and (¹⁴C)ACh were estimated as described in the text. Results are expressed in d.p.m. per mg of protein. The 0-min incubation values represented the amounts of (¹⁴C) and (³H) d.p.m. recovered in the ACh spot of the chromatograms although no ester could be synthesised in these conditions, as TCA was added just before the final incubation at 37°C. These blanks were not subtracted from the 5 and 10 min values. Each value is the mean ± s.e.m. of data obtained with a group of four samples.

incubation (Table 1). In contrast to their ability to sustain the cellular metabolic activity, ($2\text{-}^{14}\text{C}$)pyruvate molecules were unable to participate in the synthesis of ACh, that is, were unable to enter 'pool ACh'. Indeed, the lack of (^{14}C)ACh synthesis cannot be attributed to the sensitivity of the method used to measure the labelled ester, as the amounts of (^{14}C)ACh formed should represent as much as 34% of the amount of $^{14}\text{CO}_2$ formed (Fig. 1); nor can it be attributed to an impairment of the ability of the synaptosomes to synthesise ACh, as shown by (^3H)ACh sormation from ($6\text{-}^3\text{H}$)glucose (Table 1).

In conclusion, the experiments described here rule out the possibility that the anetyl moiety of ACh is derived from the acetyl CoA molecules supplying the Krebs cycle in activated acetyl groups, or from some intermediary compounds of this cycle, because an experimental situation is reported in which ($2\text{-}^{14}\text{C}$)pyruvate was used in the Krebs cycle, without any correlative formation of (^{14}C)ACh. As the PDHc specifically involved in ACh synthesis transforms ($2\text{-}^{14}\text{C}$)pyruvate which penetrates at 35°C within the nerve endings, but not ($2\text{-}^{14}\text{C}$)pyruvate already present within synaptosomes, it is tempting to speculate that this enzymatic reaction can only proceed at the level of the synaptosomal membrane. In conclusion, these observations not only allow a better understanding of the long-standing puzzle over the source of acetyl groups for ACh biosynthesis, but also challenge the established view that PDHc is exclusively localised in mitochondria.

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Both X chromosomes function before visible X-chromosome inactivation in female mouse embryos

ALTHOUGH neither X chromosome of preimplantation female mammalian embryos exhibits the two cytological signs of inactivation and dosage compensation—heteropyknosis and late replication^{1,2}—it has not been known whether both X chromosomes actually function during this period. Biochemical evidence based on increasing hypoxanthine-guanine phosphoribosyltransferase (HGPRT) activity during the mouse morula stage indicates that at least one X chromosome is functional^{3,4}. This is corroborated by the observation that mouse embryos lacking an X chromosome do not survive beyond the early cleavage stages⁵. The principal approach to determining whether both X chromosomes are functional before inactivation has been to look at the distributions of the activities of known X-linked enzymes in individual embryos^{6,7}.

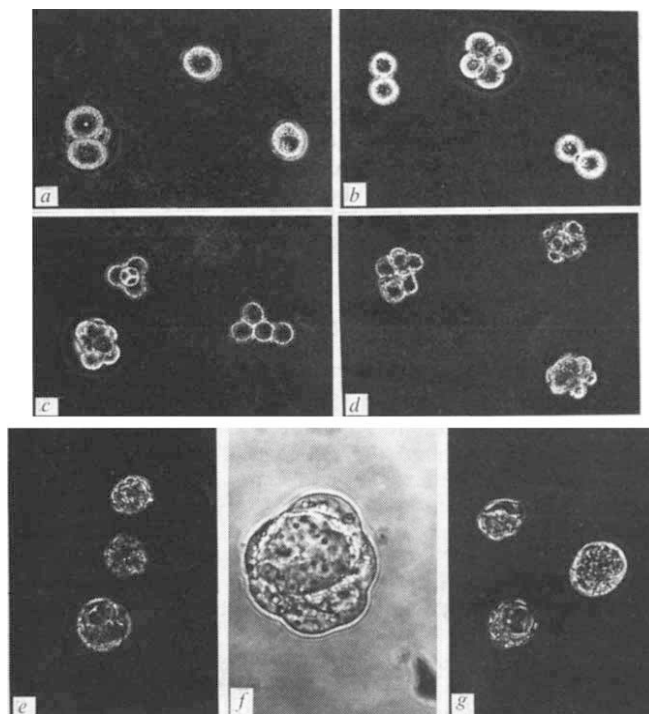


Fig. 1 Development of twin half-embryos in culture. Two-cell embryos were obtained from superovulated ICR Swiss mice and placed in modified Whitten's medium^{22,23}. The zona pellucida was softened by exposure to 0.25% Pronase (Calbiochem) in phosphate-buffered saline for 8 min at 37°C . The embryos were then washed four times in medium and transferred individually to the wells of a Terasaki microtest tissue culture plate (Falcon 3034). The zona pellucida was broken with a pipette and the blastomeres were separated. The pairs of half-embryos were then cultured at 37°C in 5% CO_2 in air under oil (10 ml Fisher light paraffin oil, previously equilibrated with medium without albumin, per plate). Within 2 h of blastulation, one half-blastocyst of each pair was placed in 2 μl of 0.01 M Tris buffer, pH 7.4, containing 0.3% bovine serum in the tip of a 1.5-ml polypropylene microsample tube (Biolab) and stored frozen at -40°C until assayed. *a*, Single blastomeres shortly after separation; *b*, half-embryos after one cleavage; *c*, half-embryos after two cleavages; *d*, half-embryos at morula stage; *e*, half-embryos at early blastocyst stage; *f*, magnified view of early half-blastocyst; *g*, early half-blastocysts. Normal embryos cultured together with half-embryos are shown for comparison. Phase contrast was used.

A bimodal distribution would be expected if dosage compensation had not yet occurred and female embryos with two X chromosomes made twice as much enzyme as male embryos with only one. However, identities of the embryos being assayed are not known, and conclusions based on activity distributions are necessarily inferential. We have circumvented this difficulty by using a method which facilitates determination of the sex of the embryonic material being assayed. This has led to the demonstration that early male and female mouse blastocysts differ twofold in HGPRT activity, a finding indicative of uncompensated X-chromosome dosage dependent gene activity before X-chromosome inactivation.

The most reliable method for embryonic sex determination in the mouse is chromosome analysis. However, because preparation of cells for karyotyping is destructive, this method cannot be applied directly to the material to be assayed. Therefore we have prepared 'half-blastocysts'⁸, by separating the blastomeres of 1.5-d two-cell embryos and culturing them in parallel. These half-embryos progressed through the normal developmental stages (Fig. 1) at a rate only slightly slower than normal whole embryos and formed twin half-blastocysts about 2.5 d after separation. One of the half blastocysts was frozen for later enzyme assay, and the other was prepared when con-

venient for chromosome analysis (Fig. 2). Both blastomeres formed half blastocysts 71% of the time, and interpretable karyotypes were obtained from 47% of the half-blastocysts used for analysis, giving an overall efficiency of 33%. The half-blastocysts used for karyotyping had 29.8 ± 2.7 (s.d.) cells, and there was no difference between male and female embryos. After determination of the sex of each half-blastocyst, frozen embryos were combined into pools of five each of the same sex and assayed for HGPRT (EC 2.4.2.8) or adenine phosphoribosyl transferase (APRT, EC 2.4.2.7) activity³. Neither the person forming the pools nor the one doing the assays knew the identities of the samples until the results were completely analysed and tabulated.

Three sets of assays of HGPRT activity were carried out on very early half-blastocysts frozen within 2 h of visible cavity formation. This stage was chosen because it is the only one for which it is possible to obtain a developmentally uniform population of embryos and because there is evidence that a bimodal distribution might not be present in later blastocysts^{7,9}. The results of these experiments are summarised in Table 1 and Fig. 3. In Fig. 3 the data are expressed relative to the mean HGPRT activities of the male pools to facilitate comparison, while the actual mean activities are listed in Table 1. In each experiment the distributions of male and female enzyme activities were different from one another, and there was virtually no overlap. In the two experiments for which sample size permits valid statistical comparisons, the male and female sample means are

significantly different from one another, with $P < 0.005$ (experiment 2) and $P < 0.025$ (experiment 3) by the Wilcoxon rank sum test (one-tailed). The combined distributions of the three experiments are also significantly different from each other ($P < 0.005$). Of greater interest, however, are the female to male ratios of mean HGPRT activities: 1.87 (experiment 1), 2.42 (experiment 2) and 2.17 (experiment 3). The female to male ratio for the three experiments combined is 2.20, quite close to the expected value of 2.00 if HGPRT activity is proportional to the number of X chromosomes in the embryo and if dosage compensation has not occurred.

Pools of very early half-blastocysts were assayed for the activity of APRT, an autosomally controlled enzyme which also increases markedly in activity in the morula stage³. As expected, the male and female values were not significantly different, with the female to male ratio being 1.19.

HGPRT activity was also determined in late half-blastocysts obtained 12–18 h after blastulation. Unlike the very early half-blastocysts, there was no significant difference between females and males and the female to male ratio was 0.95.

Comparison of the mean enzyme activities of half-blastocysts and whole blastocysts taken at approximately equivalent developmental stages indicates that HGPRT activity in the half-blastocysts is about half that in the corresponding whole blastocysts (Table 1). These results, coupled with those of the cell counts, suggest that development of the half-embryos is essentially normal at least until blastulation.

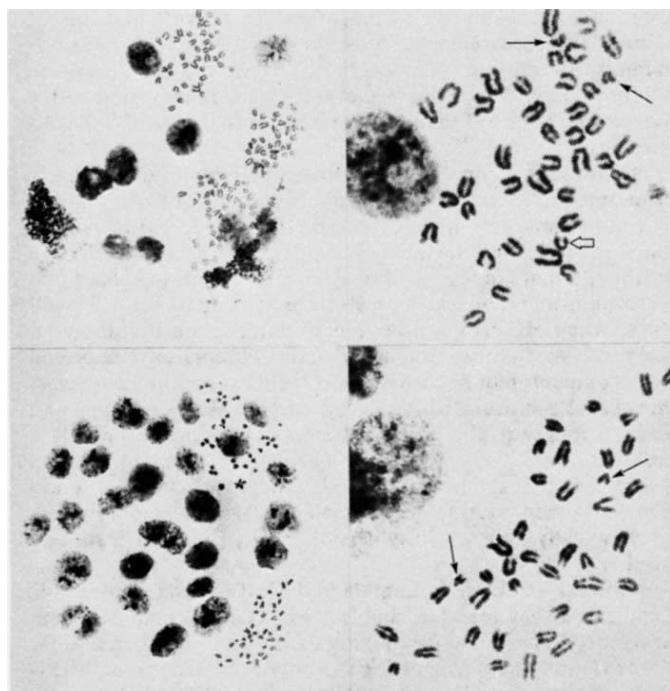


Fig. 2 Karyotype analyses of half-blastocysts²⁴. Embryos were incubated for 3–3.5 h in medium containing 10^{-8} M vinblastine sulphate (Velban, Gibco). They were then transferred to a 1% sodium citrate hypotonic solution for 12–20 min, placed on slides in a volume of 40 μ l, and fixed in fresh ethanol: acetic acid (3:2). After ageing for 3 d, slides were stained by the ASG technique²⁵ using a 60-min incubation at 60 °C in $2 \times$ SSC followed by an 8-min exposure to a 3% solution of Giemsa (Gurr's R66), pH 6.8 at room temperature. Sex determination was based on the observation of the presence or absence of the Y chromosome. Photographs were taken on Kodak SO-115 film. Upper left, spread of whole male half-blastocyst; upper right, close-up of metaphase chromosomes of single blastomere with the Y chromosome (open arrow) and two chromosomes 19 (solid arrows) marked. Lower left, spread of whole female half-blastocyst; lower right, close-up of metaphase chromosomes of single blastomere with the two chromosomes 19 marked (arrows).

Table 1 Activities of HGPRT and APRT in male and female half-blastocysts and in whole blastocysts

	Half-blastocysts			Whole blastocysts
	Male	Female	Ratio Female/Male	
HGPRT				
Very early				
Expt 1	0.99 (3)	1.86 (4)	1.87	
Expt 2	0.56 (7)	1.36 (7)	2.42	
Expt 3	1.06 (6)	2.34 (6)	2.17	3.92 (5)
Combined			2.20	
Late	4.36 (5)	4.16 (6)	0.95	12.95 (5)
				4.42 (5)
APRT				
Very early	0.96 (5)	1.15 (5)	1.19	

Enzyme activities, determined as described in the legend to Fig. 3, are expressed as fmol per h per embryo, and each value represents the mean of the number of determinations shown in parentheses. HGPRT activity of whole very early blastocysts was determined at the same time as the activities of the half-blastocysts in experiment 3; the activities of late whole blastocysts were determined at times different from those of the late half-blastocyst assays.

The activities of HGPRT and APRT in Table 1 agree reasonably well with those reported with a different analytical procedure³. However, comparison of the values obtained with the very early and the late blastocysts indicates that HGPRT activity is still increasing significantly during the very early blastocyst stage when the dosage effect is clearly demonstrable.

The reasons for the disappearance of the HGPRT dosage effect are not known although the same thing may have happened to the probable bimodal distribution of α -galactosidase noted in morulas⁷. One possibility, based on the decline of HGPRT activity after blastulation³, is that there are marked changes in the balance of HGPRT synthesis and degradation concomitant with blastulation, coupled with the onset of inactivation in the trophoctoderm^{10,11}. This would be consistent with the appearance of an allocyclic X chromosome in normal 50-cell blastocysts², the stage to which our late half-blastocysts correspond, even though other evidence indicates that

inactivation of inner cell mass X chromosomes has not been completed by the late blastocyst (4.5 d post-cleavage) stage¹².

The equalisation of male and female HGPRT activities in the later half-blastocysts could be an explanation for the failure of others to find a bimodal distribution for HGPRT activity in fully developed blastocysts^{6,9}. However, it should be noted that our own data, if grouped and analysed without regard to sex, would also not be readily interpretable as being bimodal (Fig. 4). This is because of the relatively broad and flat distribution of individual values, particularly in the female embryos. The greater spread of HGPRT activities in females than in males was observed in all experiments and is clearly evident in the combined data in Figs 3 and 4. The reasons for this greater female variability are not known.

In addition to supporting the tentative conclusion based on the α -galactosidase data⁷ and the more recent results with HGPRT^{9,14}, our results on the very early half-blastocysts are consistent with two other observations. The first is the demonstration by cell transfer and chimaeric mouse techniques that at least some of the inner cell mass cells of both early and late blastocysts have not yet undergone X-chromosome inactivation^{12,13}. The other is the finding, again based on the gene dosage

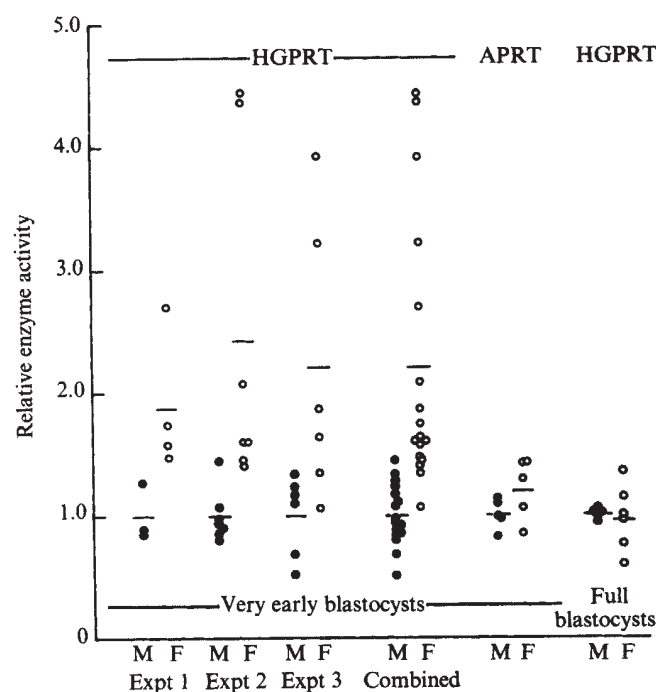


Fig. 3 Relative activities of HGPRT and APRT in male and female half-blastocysts. After determination of sex, the individual frozen half-blastocysts were pooled into groups of five of the same sex in a total volume of 10 μ l and assayed for enzyme activity as previously described³ with either ¹⁴C-hypoxanthine (59 mCi mmol⁻¹) or ¹⁴C-adenine (61 mCi mmol⁻¹) as substrate. The products of the reactions were separated on PEI cellulose sheets (Baker or Brinkmann) by Kratzer and Gartler's (personal communication and ref. 9) modification of the method of Randerath²⁶. After the PEI cellulose sheets had been pre-washed by ascending chromatography in water, the reaction products were spotted over the appropriate markers and run in water in the same direction as the pre-wash. After drying, the hypoxanthine or adenine spots were located under ultraviolet light and cut off the sheet. The sheet was reversed and run progressively in 0.5 M, 2 M and 4 M sodium formate, pH 3.4. After drying, the IMP or AMP, ADP and ATP spots were located under ultraviolet light, cut out and counted in a Beckmann LS-250 liquid scintillation spectrometer in 10 ml of an Omnifluor-toluene counting solution. To facilitate comparisons among experiments and pooling of data, the results of each experiment were normalised to a mean male enzyme activity of 1. M, male; F, female.

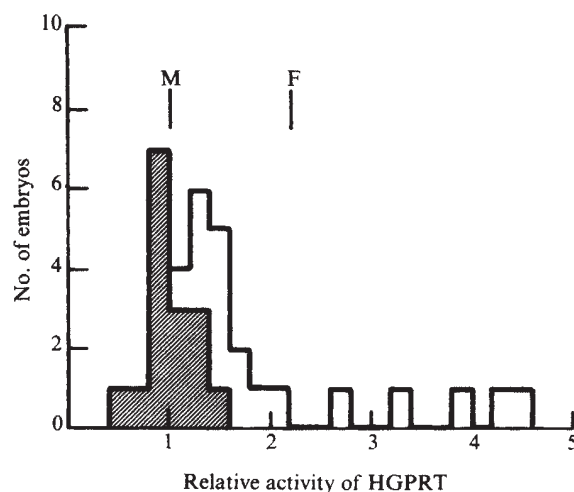


Fig. 4 Distribution of activities of all half-blastocysts. The combined HGPRT data in Fig. 3 have been plotted as a histogram showing the distribution of enzyme activity in 0.2-unit increments. The part of the distribution occupied by the male embryos is shown by the cross-hatching, and the male and female means are marked above. In spite of the almost complete separation of values in Fig. 3, plotting as a histogram in the absence of knowledge of sex would obscure the male-female difference.

approach, that both X chromosomes are active¹⁵ in a line of cultured teratocarcinoma cells which correspond developmentally to embryonic ectoderm^{16,17}. These cells, if allowed to differentiate *in vitro*, exhibit X-chromosome inactivation with a reduction from 2 to 1 in the XX to XO ratios of several X-linked enzymes.

The present picture of X-chromosome activity during early development in the mouse can be summarised as follows. During oogenesis and oocyte growth both X chromosomes function^{4,18-20}, but the male X apparently becomes inactive during spermatogenesis²¹. Shortly after fertilisation at least one X chromosome is operative in all embryos, and by the 8-16-cell stage, when HGPRT synthesis is initiated, both the maternal and paternal X chromosomes are active in females. The period of uncompensated X-chromosome activity continues at least through the morula to the very early blastocyst stage and perhaps longer in the inner cell mass. Therefore, even if it is assumed that X-chromosome inactivation is complete, an assumption that can be questioned¹⁰, there is certainly a period in development during which male and female embryos are biochemically and possibly physiologically different from one another.

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HGPRT activity changes in preimplantation mouse embryos

To compensate for unequal doses of genes on the X chromosomes of males and females, one of the X chromosomes in the somatic cells of mammalian females is inactive¹. This inactivation occurs early in development, although the exact time is unknown². Before X-chromosome inactivation, and in the absence of other dosage compensating mechanisms, female embryos with two X chromosomes would be expected to have twice as much activity for an X-linked enzyme as male embryos with only one X chromosome. In a litter with approximately an equal number of male and female embryos, the distribution of enzyme activity should have two equal-sized peaks separated by a factor of two. The change from a bimodal to unimodal distribution would indicate that X-chromosome inactivation had occurred. Early in development, the X-linked enzymes α -galactosidase (α -gal)³ and hypoxanthine guanine phosphoribosyltransferase (HGPRT)^{4,5} are both derived from embryonic gene activity. α -gal was found to have a bimodal distribution at the morula stage³. For HGPRT, Monk and Kathuria⁶ found no bimodality at either the eight-cell or blastocyst stages, however further analysis revealed bimodality at certain stages⁷. Epstein *et al.*⁸ have found that females have twice as much HGPRT activity as males in early blastocysts. We present here evidence for the activity of both the maternal and paternal X chromosomes by the eight-cell stage, with inactivation initiated at blastulation.

The distribution of HGPRT activity was determined for litters at several stages throughout the preimplantation period of the mouse. Embryos obtained from superovulated (BALB/c $\times$ S1)F₁ females mated to S1 males (a strain selected for large litters⁹) were assayed individually for HGPRT activity. Frequency distributions of HGPRT activities for embryos from the same litter were tested for bimodality against unimodality on a computer program. The program computes the maximum likelihood for a distribution assuming first unimodality, then bimodality. Twice the difference in the logarithm of the maximum likelihood estimates yields a χ^2 value which indicates whether a bimodal curve fits the distribution significantly better than a unimodal curve. The program also calculates the mean and proportion of embryos in each peak of the bimodal curve.

The results are presented in Table 1. Before the eight-cell stage, distributions are unimodal, presumably because the activity is due solely to maternal enzyme. At the eight-cell stage, the distributions are clearly bimodal (Fig. 1). The two-peaks have approximately equal proportions of embryos, and the mean of the higher peak is twice the value of the lower peak (ratios of higher: lower are 2.04, 2.07, 2.08). The simplest interpretation of the bimodality is that the lower peak represents the activity of male embryos, and the higher peak

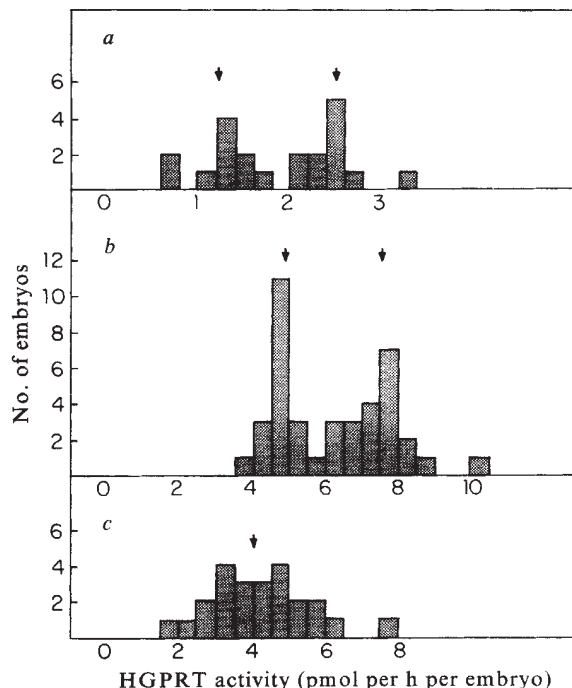


Fig. 1 Distribution of HGPRT activity per embryo is shown for three litters at different developmental stages: *a*, eight-cell (litter no. 294, day 2 at 1400 h); *b*, late blastocyst (litter no. 278, day 3 at 2000 h); *c*, very late blastocyst (litter no. 280, day 4 morning). The arrows indicate the means of each distribution. Pregnant mares' serum (Organon, 8 IU) was injected at 1500 h, followed 48 h later by an injection of human chorionic gonadotropin (Ayerst, 5 IU). Vaginal plugs were checked the next morning, which was called day 0 of development. Embryos were removed into rinsing solution (saline plus 1% HGPRT buffer (Tris-HCl, pH 7.4, 50 mM; MgCl₂, 5 mM; NaN₃, 10 mM), bovine serum albumin, 1 mg ml⁻¹), washed five times in rinsing solution plus bovine serum albumin (3 mg ml⁻¹) morphologically staged under a dissection microscope, and individually transferred in 10 μ l of the final wash to a glass tube. After freeze-thawing, embryos were incubated for 2 h (assay is linear for at least 4 h) at 37 °C in a total incubation volume of 35 μ l (10 μ l embryo, 25 μ l HGPRT buffer plus 5.5 mM phosphorylribose-pyrophosphate and 4.8 mM 8-¹⁴C-hypoxanthine (New England Nuclear) containing 440 $\times$ 10³ c.p.m.). After the reaction had been stopped by freezing, the product, inosinic acid (IMP), was separated from unreacted hypoxanthine by thin-layer chromatography. Pre-washed (H₂O) poly-ethylenimine (PEI)-cellulose sheets (Baker or Macherey-Nagel) were spotted with the incubation mixture and subjected to ascending chromatography with H₂O to wash out the unreacted hypoxanthine. The part of the sheet containing hypoxanthine was cut off and discarded. Next the sheets were washed in water for 20 min and methanol for 10 min and chromatographed in the opposite direction in a sodium-formate buffer²¹ to isolate the product. The IMP spot was cut out and counted in Omnifluor in a liquid scintillation counter. To calculate HGPRT activity, counts for controls, which contained 10 μ l of the final embryo wash and were treated identically to samples, were subtracted from the counts for the samples. Recovery of IMP from the chromatographic system was nearly 100%. To correct for slight differences between sheets, HGPRT activities were normalised within litters. The frequency distributions were tested for unimodality/bimodality using an adaptation of a computer program²² for the maximum likelihood analysis of mixtures of normal distributions²³.

represents the activity of female embryos. According to this interpretation, both maternal and paternal X chromosomes are active by the eight-cell stage. It has been reported that paternal genes on both the X chromosome and autosomes are expressed by this stage^{3,10-13}. Bimodality for HGPRT activity additionally means that lack of dosage compensation for HGPRT during very early development is not detrimental to the embryo.

Table 1 Distribution of HGPRT activity per embryo

Stage	Litter no.	No. of embryos	χ^2	Peak means			Peak proportions	
				Lower	Higher	Ratio ($\frac{\text{upper}}{\text{lower}}$)	Lower	Higher
4-8-cell	282	15	0	1.48	1.62	1.10	0.73	0.27
	286	12	1.92	1.17	1.54	1.32	0.46	0.54
	297	14	5.39	0.79	1.06	1.34	0.85	0.15
		Sum	7.31*					
8-cell	264	18	5.06	1.29	2.68	2.07	0.72	0.28
	291	25	11.76	1.06	2.20	2.08	0.58	0.42
	294	21	7.58	1.23	2.50	2.04	0.52	0.48
		Sum	24.40†					
Compact 8-cell	292	15	1.39	1.14	2.22	1.95	0.66	0.34
	295	15	2.40	1.46	2.33	1.60	0.53	0.47
		Sum	3.79*					
Morula	267	16	6.59	1.75	3.93	2.24	0.37	0.63
	274	24	3.85	3.43	6.11	1.78	0.66	0.34
	288	28	0.56	2.89	6.16	2.13	0.72	0.28
		Sum	11.00†					
Early-mid-blastocyst	262	13	0.95	6.13	10.19	1.66	0.63	0.37
	271	32	6.12	4.46	7.83	1.76	0.53	0.47
	275	30	7.51	6.06	10.00	1.65	0.47	0.53
	287	16	0	5.76	5.97	1.04	0.63	0.37
		Sum	14.58†					
Late blastocyst	277	49	13.96	5.63	7.96	1.41	0.53	0.47
	278	40	12.42	4.89	7.59	1.55	0.53	0.47
	293	21	14.44	6.45	10.20	1.58	0.56	0.44
		Sum	40.82‡					
Very late blastocyst	276	16	2.18	5.61	8.84	1.58	0.67	0.33
	280	24	0.06	3.95	6.64	1.68	0.91	0.09
	289	28	0.31	4.40	7.00	1.59	0.67	0.33
	290	15	3.61	5.18	6.96	1.35	0.48	0.52
		Sum	6.16*					

Mean HGPRT activities are expressed as pmol per h per embryo.

* $P > 0.10$.

† $0.05 < P < 0.10$.

‡ $P < 0.001$.

In late blastocysts, there is also marked bimodality, with approximately equal proportions in the two peaks. However, at this stage, the mean of the higher peak is only about 50% greater than the mean of the lower peak (ratios: 1.41, 1.55, 1.58). In very late blastocysts, just before implantation, the distributions are again unimodal. The switch from bimodality to unimodality (that is convergence of two peaks into one) is what is expected for X-chromosome inactivation. The consistent decrease in separation of the peaks from 2:1 to 1.5:1 begins between the morula (ratios: 1.78, 2.13, 2.24) and early-mid blastocyst stages (ratios: 1.04, 1.65, 1.66, 1.76). Thus inactivation seems to occur between the morula and early blastocyst stages. The accuracy of this estimate depends on the rate of turnover of HGPRT. Judging from the decrease in HGPRT activity between late and very late blastocysts, the turnover must be fairly rapid.

The litters between the eight-cell and late blastocyst stages show some tendency toward bimodality, although not the marked bimodality exhibited by late blastocysts and eight-cell embryos. The absence of marked bimodality could be due to increased variability of embryos within a litter. During this period of development, HGPRT activity increases most rapidly (Table 1, ref. 4). Variability may also result if X chromosome inactivation occurs at different times in different cells.

The estimate of the time of inactivation given here is consistent with cytological estimates. The detection of sex chromatin¹⁴, prophase heteropycnosis¹⁵ and replication asynchrony^{15,16} have previously been reported to occur around the blastocyst stage in the mouse. Genetic approaches on the other hand have produced later estimates for the time of inactivation. Studies on the extent of variegation in females heterozygous for an X-linked marker^{17,18}, or from chimaeras produced by marked single-cell injections into recipient blastocysts¹, indicate that X-chromosome inactivation occurs at or after implantation. Our estimate that it occurs at blastulation

can be reconciled with the cytological and genetic estimates if most of the HGPRT activity is contributed by the trophectoderm and inactivation occurs earlier in the trophectoderm. X-chromosome inactivation in the trophectoderm is known to differ from that in the cells that will make up the fetus, inasmuch as inactivation in the trophectoderm is not random^{19,20}.

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Polymerisation of red cell membrane protein contributes to spherocytosis shape irreversibility

THE discocyte-spherocytosis transformation of red cells undergoing ATP depletion and calcium accumulation is reversible after restoration of normal ATP and calcium levels or exposure of cells to stomatocytogenic agents such as cationic anaesthetics¹⁻³. This transformation becomes irreversible after a prolonged incubation without glucose or an introduction of high calcium concentrations into the cells.⁴ In our studies of membrane protein composition of metabolically depleted red cells, we have noted that aerobically ATP-depleted erythrocytes contained a $>1 \times 10^6$ -dalton reducible membrane protein polymer which was selectively enriched in spectrin, the major protein at the cytosol membrane interface⁵. We suggested that the formation of this complex was due to changes in the assembly of spectrin in ATP-depleted red cell membranes to form closer contacts, allowing a spontaneous crosslinking of the nearest spectrin neighbours through disulphide couplings⁵. Another polymer differing from that in ATP-depleted red cells by an absence of cleavage with reducing agents was recently noted in fresh red cells, into which Ca^{2+} (>0.5 mM) was introduced by ionophore A23187 (ref. 6); it has been attributed to a crosslinking of γ -glutamyl ϵ -lysine residues of spectrin and other membrane proteins, catalysed by a Ca^{2+} -activated cytoplasmic transglutaminase^{6,7}. As both the above membrane protein polymers were found in cells which transformed into spherocytosis shape, we have studied and report here results which show that such spontaneous membrane protein crosslinking contributes to a permanent fixing of the red cells to their abnormal shape.

To study the reducible polymer of aerobically ATP-depleted red cells we incubated fresh red cells under 100% O_2 or N_2 in a glucose-free isotonic medium containing 5 mM KCl, 2 mM MgCl_2 , 50 mM glycyl glycine (pH 7.4) and NaCl up to

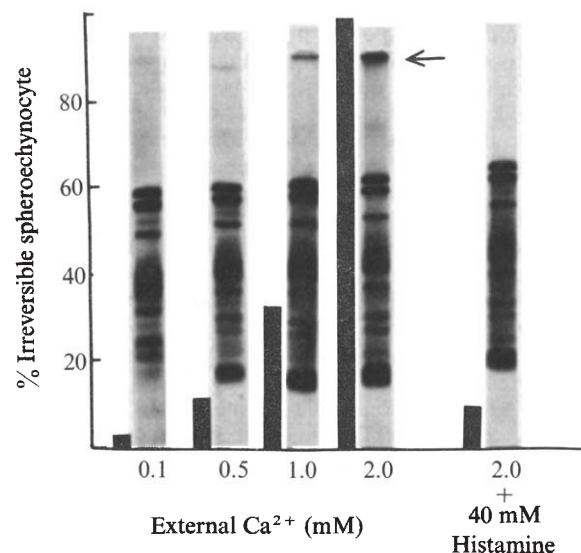


Fig. 2 Ca^{2+} concentration dependence of the formation of the nonreducible membrane protein polymer (arrow) and the % of irreversible spherocytosis (solid bars). Last PAGE: 40 mM histamine was added before A23187 and 2 mM Ca^{2+} .

290 mOsm for 19 h at 37 °C. Under either N_2 or O_2 , $>95\%$ of cells converted to type III echinocytes or spherocytosis, classified according to ref. 8. ATP was subsequently restored by incubation (under N_2) with adenine, inosine and glucose⁵. The experiments involving the Ca^{2+} -dependent nonreducible polymer were carried out by suspending fresh red cells in the above isotonic solution containing Ca^{2+} (0.1–5 mM). Subsequently, A23187 (0.01 mM) was added and samples incubated for 30 min at 37 °C to induce echinocyte transformation. A23187 was then removed by washing with isotonic 0.5% albumin and cells incubated with adenine, inosine and glucose for 16 h at 37 °C to restore normal levels of intracellular ATP and calcium. At times indicated below, we have

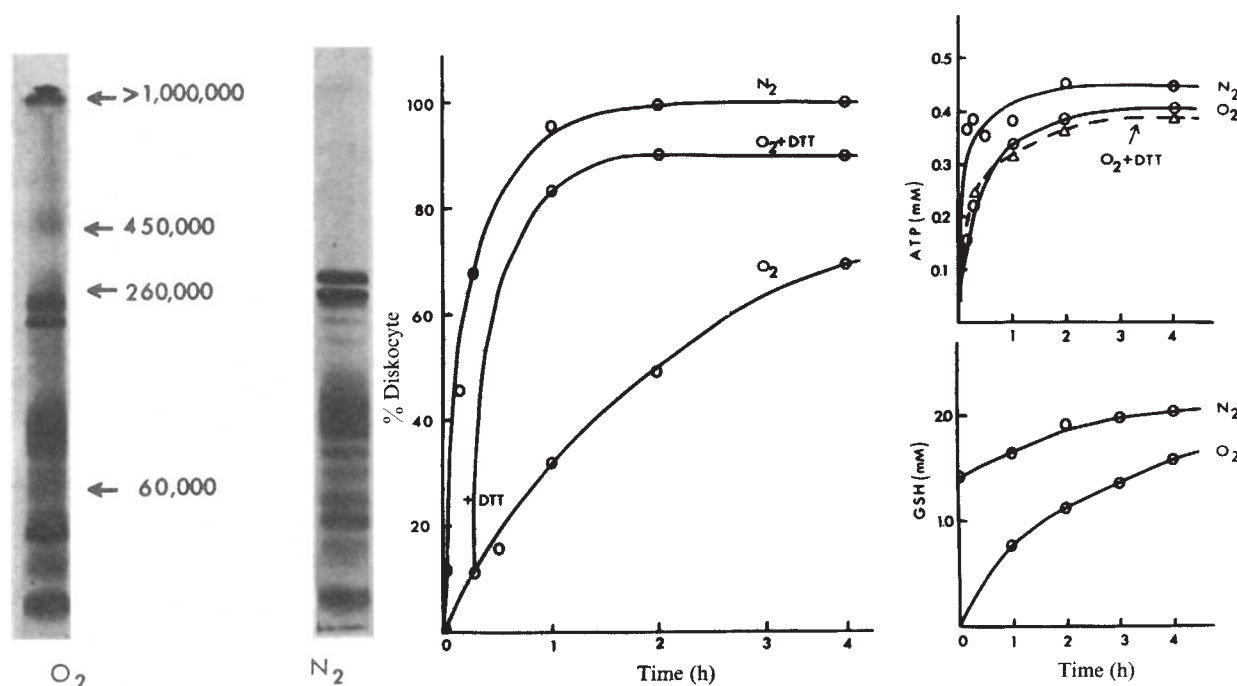


Fig. 1 Membrane protein composition, recovery of biconcave shape, ATP and GSH levels of ATP-depleted erythrocytes during a subsequent incubation with glucose, adenine and inosine. Red cells were depleted by 19 h incubation (37 °C) under N_2 , O_2 , or O_2 followed by an addition of 10 mM DTT at the onset of ATP rejuvenation. Percentage of discocytes, GSH and ATP (mmol l^{-1}) are plotted as a function of rejuvenation time.

determined red cell shape by phase contrast and scanning electron microscopy³, ATP, reduced glutathione (GSH) (ref. 9) and membrane protein composition by polyacrylamide gel electrophoresis (PAGE, ref. 10), and also the amount of calcium introduced and subsequently extruded from the cells. The latter was measured by ⁴⁵Ca. The net amounts were calculated from red cell disintegrations per minute and the specific activities of external ⁴⁵Ca (ref. 11). As the amounts of Ca²⁺ introduced into the cells exceeded 0.5 mM (Fig. 3), it was not necessary to correct for equilibration of ⁴⁵Ca with an exchangeable red cell calcium pool, which in normal cells does not exceed 0.003 mmol per l of cells¹².

The effect of the reducible spectrin-rich polymer in aerobically ATP-depleted cells on their restoration of discoidal shape after ATP repletion is shown in Fig. 1. Echinocytes, produced by aerobic ATP depletion, which contained the polymer exhibited a markedly decreased propensity to restore discoidal shape when compared with echinocytes induced by anaerobic ATP depletion; the latter cells failed to exhibit this polymer. The restoration of biconcave shape of aerobically ATP-depleted red cells was markedly improved by exposing them to reduction with dithiothreitol (DTT), which completely dissociated this polymer⁵. The restoration of biconcave shape further correlated with the level of GSH. In anaerobically (N₂) ATP-depleted erythrocytes, there was only a slight decrease in GSH at the end of ATP depletion. This contrasted with a near absence of GSH in aerobically ATP-depleted red cells; GSH restoration during a subsequent ATP repletion coincided with an increased percentage of biconcave cells during incubation (Fig. 1, right) and a partial dissociation of the reducible spectrin-rich polymer (to 40% of preincubation amounts at two hours and 37 °C (not shown)). In contrast, there were no major differences in the kinetics of the restoration of ATP levels between aerobically and anaerobically ATP-depleted red cells. These data suggest that the delayed and incomplete reversal of aerobically ATP-depleted spherocytes to discocytes during ATP repletion is related to an oxidation-induced, reversible membrane protein crosslinking, which stabilises the altered spectrin arrangement in such cells.

The role of the nonreducible polymer produced by introduction of Ca²⁺ into the cells is indicated in Fig. 2. Red cells

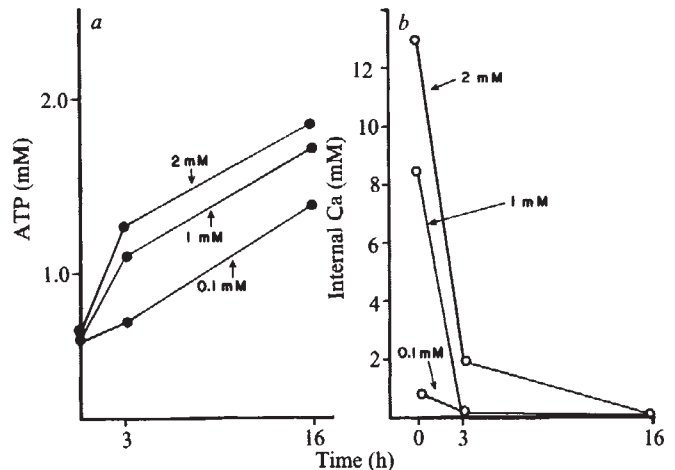


Fig. 3 Restoration of ATP level (a) and calcium extrusion (b) from red cells previously exposed to 0.1, 1.0 and 2 mM Ca²⁺ plus A23187 during a subsequent incubation (37 °C) with glucose, adenine and inosine. Arrows indicate external Ca²⁺ concentration.

exposed to progressively increasing Ca²⁺ concentrations exhibited increased amounts of this complex (Fig. 2) which remained unchanged after a subsequent ATP repletion and calcium extrusion (not shown). This coincided with a progressive increase in the fraction of cells which remained irreversibly spherocytes (Figs 2, 4) in spite of restoration of their normal ATP and calcium levels (Fig. 3). Addition of histamine (40 mM), a transglutaminase inhibitor⁶, inhibited the formation of the polymer (Fig. 2) and completely prevented the formation of irreversible spherocytes (Figs 2, 4).

We conclude that the discocyte-spherocyte transformation of ATP-depleted or calcium-enriched erythrocytes becomes irreversible when the altered assembly of spectrin and other membrane proteins of these cells is stabilised by spontaneous membrane protein crosslinking. Such crosslinking can result both from reversible intermolecular disulphide couplings (aerobically ATP-depleted red cells) or from permanent protein crosslinking catalysed by a Ca²⁺-activated red cell trans-

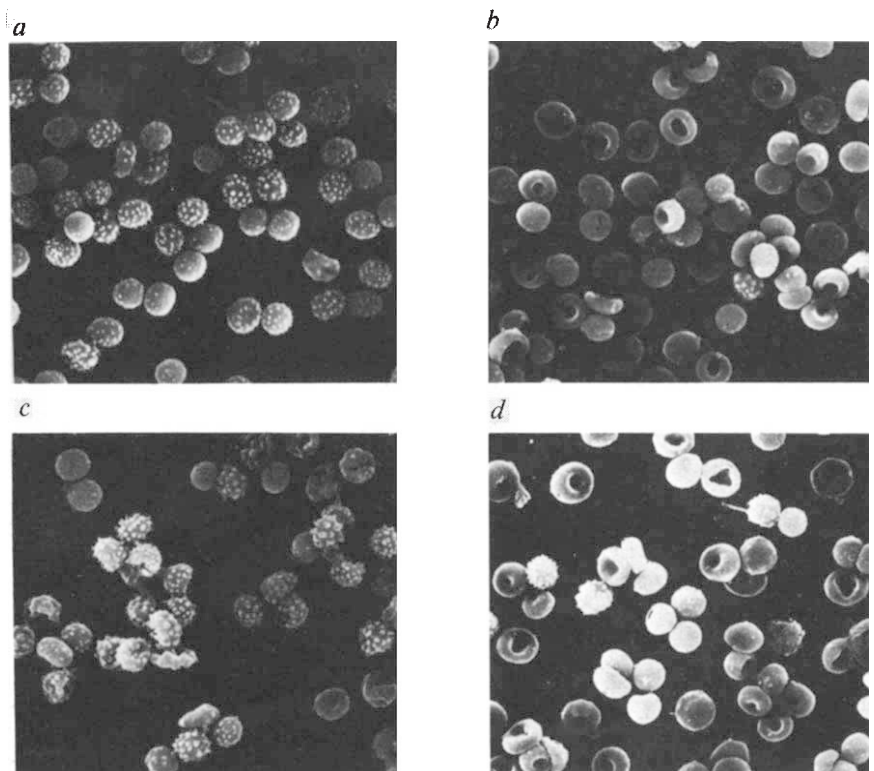


Fig. 4 Scanning electron micrographs of red cells treated with 0.1 mM Ca²⁺ and A23187 for 1 h (a), 0.1 mM Ca²⁺ and A23187 followed by 17 h incubation with glucose, adenine and inosine (b), 2 mM Ca²⁺ and A23187 followed by incubation as above (c), and 2.0 mM Ca²⁺ + A23187 + 40 mM histamine followed by incubation (d).

glutaminase. It remains to be investigated whether this *in vitro* phenomenon is relevant to the stabilisation of abnormal red cell shape in certain pathological conditions *in vivo*.

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Photosensitised electron transport across phospholipid vesicle walls

As a means of solar energy conversion, we are interested in the photosensitised decomposition of water with pigmented lipid bilayer membranes that have O_2^- and H_2 -evolving catalysts at opposing membrane surfaces. Our present work toward this goal involves the investigation of light-induced electron transfer reactions between aqueous solutions of electron donors and acceptors that are on opposite sides of single-lamella liposomes (vesicles) with incorporated surfactant photosensitisers. Related experiments with planar bilayer membranes¹ or liposomes²⁻⁴ using chlorophyll as the photosensitiser have been reported, but it is not clear that energy storage has been achieved in these systems. A possible exception is the photo-oxidation of water with vesicles that contain chlorophyll and trapped aqueous ferricyanide³, although attempts to duplicate this experiment have been unsuccessful⁵. We report here that we have observed the photosensitised reduction of methylviologen (1,1'-dimethyl-4,4'-bipyridinium²⁺, abbreviated to MV²⁺) dissolved in the exterior aqueous phase of a phospholipid vesicle dispersion containing a surfactant analogue of *tris*-(2,2'-bipyridine)ruthenium²⁺ when ethylenediamine-*N,N,N',N'*-tetraacetate (EDTA) is dissolved in the enclosed aqueous phase of the vesicles.

The net reaction, shown in Fig. 1, involves the irreversible oxidation⁶ of EDTA; the reaction is endoergic by approximately 20 kilocalories per mol EDTA consumed. This reaction is sensitised in aqueous solutions by the photoexcited triplet state of *tris*-(2,2'-bipyridine)ruthenium²⁺ (ref. 7). Representative experiments are illustrated in Fig. 2.

The vesicles described here contained phyloquinone (vitamin K₁, a hydrogen carrier⁸), hexadecylviologen (1,1'-dihexadecyl-4,4'-bipyridinium²⁺), and decachloro-*m*-carborane (a proton carrier⁹) in addition to egg yolk phosphatidylcholine and (*N,N'*-di(1-hexadecyl)-2,2'-bipyridine-4,4'-dicarboxamide)-*bis*-(2,2'-bipyridine)ruthenium²⁺ (abbreviated to Ru). The vesicle dispersions were prepared (see Fig. 3) by rapidly injecting¹⁰ (using a syringe) 0.3 ml of an ethanol-dimethylformamide solution of the vesicle components into 6 ml of a vigorously stirred aqueous solution composed of buffered 0.5 M EDTA (as its ammonium salt). The buffer was

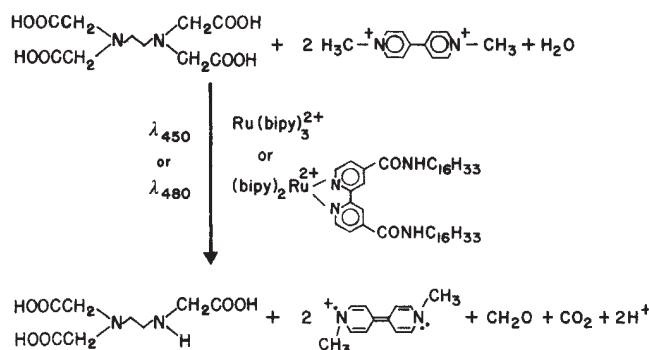
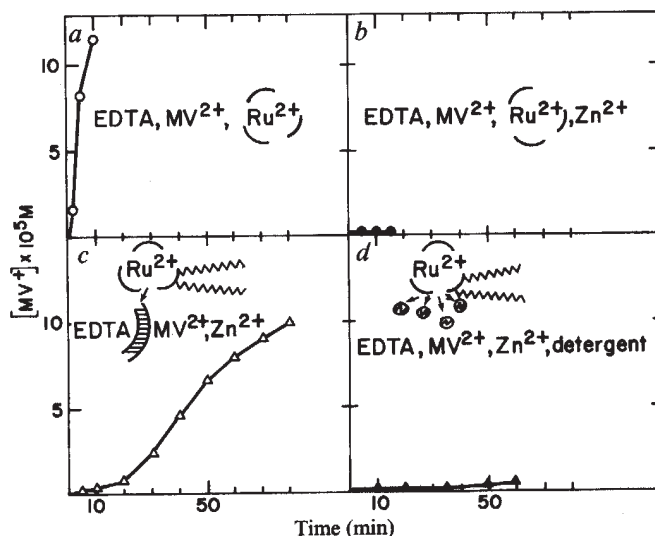


Fig. 1 Net reaction for the photosensitized reduction of methylviologen and oxidation of EDTA.

1 M ammonium acetate, 0.1 M NaCl and 0.1 M KCl, pH 7. The composition of the organic solution of the vesicle components is listed in Fig. 3. Untrapped EDTA was removed by gel filtration of 2 ml of the vesicle dispersion over a column (11 × 185 mm) of Sephadex G-25 that had been equilibrated with buffer. The eluted vesicle fraction (2.5 ml) was transferred to a rubber serum-stoppered glass tube that contained a Teflon-coated magnet, 12.5 μl 0.20 M aqueous methylviologen dichloride and 25 μl 1.0 M zinc acetate. Air in the magnetically stirred sample was replaced with argon by four cycles of evacuation followed by bubbling with argon. The vesicle dispersion was transferred (using a syringe) to a glass cuvette with a 1-cm pathlength and bubbled briefly with argon before the stopcock at the top of the cuvette was closed. The cuvette was immersed in a glass container with 10 ml water and irradiated with a collimated beam from a 900-W xenon arc lamp. Light

Fig. 2 Change in methylviologen radical cation (MV^{•+}) concentration with time of exposure to high intensity visible light in homogeneous (a and b) and micellar (c and d) aqueous solutions flushed with argon. The concentration of MV^{•+} was calculated from the increase in A_{603} ($\epsilon = 12,400 \text{ M}^{-1} \text{ cm}^{-1}$). a, Buffered (pH 7) aqueous solution contained 0.05 mM *tris*-(2,2'-bipyridine)ruthenium²⁺, 0.5 mM methyl viologen (MV²⁺) and 1 mM EDTA. b, The solution had the same composition as in a except that 2 mM Zn²⁺ was also present. No MV^{•+} was detected after 15 min of illumination. c, 0.5 M aqueous EDTA was trapped in phospholipid vesicles that contained a surfactant analogue of *tris*-(2,2'-bipyridine)ruthenium²⁺, 1 mM MV²⁺ and 10 mM Zn²⁺ were added to the vesicles after the removal of untrapped EDTA by gel filtration. See text for details. d, Vesicles with trapped 0.5 M EDTA were prepared as c, but in this case 18 mM 3-(dimethylhexadecylammonio)propane-1-sulphonate was added with 1 mM MV²⁺ and 10 mM Zn²⁺ to solubilise the vesicles and release the trapped EDTA.



with wavelengths longer than 600 nm and shorter than 420 nm was removed using aqueous cupric sulphate and Corning 3-73 filters. Absorption spectra of the sample were recorded before illumination and after intervals of steady illumination. The optical density of the vesicle dispersion at the visible absorption maximum of Ru (480 nm) was 0.8. The concentration of viologen radical produced was estimated using the value of $12,400 \text{ M}^{-1} \text{ cm}^{-1}$ for its extinction coefficient at 603 nm (ref. 11).

Approximately 10^{-4} M of viologen radical was present in the vesicle dispersion after 80 min of illumination (Fig. 2c). As the concentration of the hexadecylviologen was about 10^{-5} M , it is clear that MV^{2+} , added after vesicle formation, was being reduced. The concentration of Ru (about $8 \times 10^{-5} \text{ M}$) changed by less than 5%, so the complex was acting as a photocatalytic agent. No reduced viologen was observed after 60 min of illumination in the control experiment without EDTA.

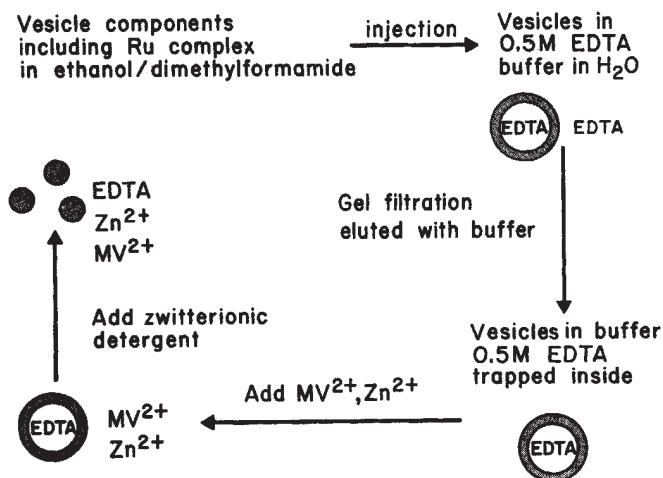


Fig. 3 Schematic diagram of the method used for preparing the vesicle dispersions. The composition of the injected organic solution was as follows: phosphatidylcholine 42 mM; (*N,N'*-di(1-hexadecyl)-2,2'-bipyridine-4,4'-dicarboxamide)*bis*(2,2'-bipyridine)ruthenium²⁺ 2.0 mM; phylloquinone 2.0 mM; hexadecylviologen 0.20 mM; decachloro-*m*-carborane 0.20 mM; 80% ethanol, 20% dimethylformamide (v/v). The volume of the injected solution was 5% of the aqueous solution volume. Other details are given in the text.

To insure that EDTA trapped inside the vesicles was the source of electrons for the MV^{2+} reduction, 10 mM Zn^{2+} had been added to the gel-filtered vesicles. The inhibitory effect of Zn^{2+} on the photosensitized reaction in aqueous solution was demonstrated using *tris*-(2,2'-bipyridine)ruthenium²⁺ as the photosensitizer (Fig. 2b). The rate of MV^{+} formation in the presence of 1 mM EDTA and 2 mM Zn^{2+} was no more than 1% of the rate with 1 mM EDTA alone. The bulk concentration of EDTA, trapped as its 0.5 M aqueous solution in phosphatidylcholine vesicles that had been gel filtered and dissolved with detergent, was estimated to be $1.5 \pm 0.3 \text{ mM}$, as determined spectrofluorometrically using the Cu^{2+} complex of calcein blue as indicator. Therefore the amount of zinc ion in the vesicle dispersion was well in excess of the amount of EDTA outside the vesicle walls that was not removed by gel filtration or that may have escaped from the vesicle interiors. Finally, the impermeability of phospholipid vesicles to MV^{2+} was demonstrated by comparing the concentration of MV^{2+} (determined spectrophotometrically) in vesicle dispersions with trapped 0.20 M MV^{2+} that had been gel filtered once, and then a second time 5 min or 2 h after the first gel filtration.

Thus, we have carried out a photosensitized oxidation-reduction reaction, up a free energy gradient, with the reactants contained in separate compartments joined by a vesicle wall through which a net transfer of electrons and protons has taken place.

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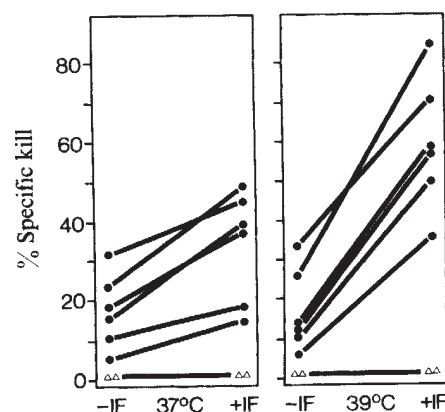
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The actions of interferon are potentiated at elevated temperature

INTERFERON was discovered¹ because of its antiviral properties, but interferon preparations have other biological activities. These include inhibition of transplanted tumours *in vivo*² and of cell multiplication *in vitro*^{3,4}, as well as regulatory roles in the immune response⁵. The effects of interferon on immune responses *in vivo* and *in vitro* have mostly been known as inhibitory, in particular on B-cell functions, whereas we have discovered augmented killer T-cell generation *in vitro* in the presence of interferon⁶. As interferon is produced *in vivo* in the course of acute viral infections (or after vaccination) at a stage when the temperature of the host is elevated⁷⁻⁹ we have

Fig. 1 Enhancing effect of interferon on CML in MLR at different temperatures. Six MLR-CML temperature experiments are shown. Killer cells generated in MLR in the absence of interferon (-IF) and in the presence of 500 U ml^{-1} (+IF) are compared at 37° and 39°C. ●, specific release by allogeneic target-cells; △, release by autologous target cells. The final effector/target cell ratio varied between experiments from 15:1 to 25:1. Viable cell recovery from MLR ranged from 76 to 122% of the input cells at 37°C and from 73 to 127% at 39°C.



investigated the influence of increased temperature on some of the effects of interferon. We report here that elevated incubator temperatures potentiate the antiviral activity, the killer-augmentory properties and the growth-inhibitory action of interferon on a lymphoblastoid cell line (Daudi).

Partially purified human leukocyte interferon with a specific activity of 1×10^6 interferon units per mg of protein was obtained from K. Cantell, Helsinki, and highly purified interferon (specific activity $>1 \times 10^8$ units per mg of protein) was prepared in our laboratory from crude Sendai virus-induced leukocyte interferon¹⁰. The interferon units (U) are referred to the 69/19B reference preparation provided by the British MRC. Both interferon preparations gave similar results in the experiments described here.

The influence of temperature on the antiviral activity of interferon was established by titrating the same leukocyte interferon sample at 37°, 38°, 39° and 40°C, using the usual plaque reduction method on human embryonic lung (HEL) cells¹¹. Table 1 shows that the antiviral activity of interferon was increased more than threefold when the temperature (of the incubator) was increased from 39° to 40°C. Similar results were obtained using several interferon preparations, including leukocyte and fibroblast interferon, of different purities. At lower temperatures (35° and 36°C) the titre of interferon was the same as found at 37°C. Mock interferon preparations had no activity.

The effect of interferon on lymphocyte killer activity was assayed using a one-way mixed lymphocyte reaction (MLR), as described previously⁶. Blood samples were obtained from healthy volunteers. Lymphocytes were separated on Ficoll-Isopaque gradients¹² and MLRs were set up in microtitre plates at different temperatures, different stimulator/responder cell ratios, and in the presence or absence of interferon. At different times (mostly 6 d) after initiation of the cultures, cell-mediated lympholysis (CML) activity was measured. For this the cultures were re-fed with interferon-free medium, and 10^4 concanavalin A-stimulated, ^{51}Cr -labelled lymphoblasts of responder (autologous control) or stimulator origin were added. After incubation for 4 h at 37°C, ^{51}Cr -release was measured. When assayed on day 6 or earlier, viable cell recovery from MLR cultures at temperatures of 39°C and below with or without interferon were identical although thymidine uptake was consistently inhibited, in the cultures with interferon, as described previously⁶. CML was performed by direct

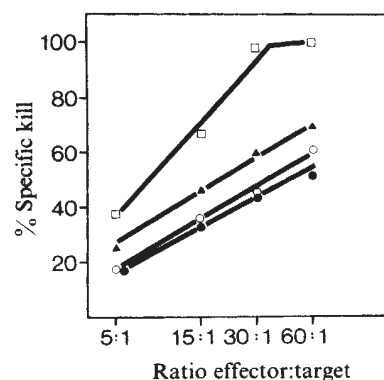


Fig. 2 Titration of the number of effector cells in CML with fixed numbers of target cells. Effector cells generated with or without interferon at two temperatures are compared. $\square$, 39°C with interferon; $\blacktriangle$, 37°C with interferon; $\circ$, 39°C without interferon; $\bullet$, 37°C without interferon.

addition of 10^4 target cells to such cultures after the change of medium. This procedure ensured the same effector target cell ratio in the individual experiments.

Titration of interferon from 0 to 10,000 U ml⁻¹ in MLRs showed that at 37° and 39°C enhancement of the CML product was optimal at the same interferon concentrations—in the range 200–1,000 U ml⁻¹. Figure 1 shows the results of six CML experiments in different allogeneic combinations with 500 U ml⁻¹ at 37° and 39°C. Specific kill in cultures without interferon was very similar at the two temperatures, whereas the interferon-treated cultures gave rise to an enhanced killer cell product, the effect being very pronounced at 39°C, corresponding to a threefold mean increase in the percentage kill. Autologous target cells were lysed to a negligible extent.

Temperatures from 35° to 41°C were tested and the maximum enhancement of CML activity was generated at 38°C and 39°C. At 40°C and higher temperatures viable cell recoveries from MLR were considerably lower, but whenever recovered cells were assayed, the killer cells generated in the presence of interferon had considerably greater potency compared with the untreated and mock interferon-treated controls.

Table 1 Effect of incubation temperature on antiviral activity of interferon in HEL cultures challenged with VSV

Dilution of interferon*	No. of VSV plaques† obtained at temperatures of			
	37°C	38°C	39°C	40°C
1/2,000	0-0-0 (0)	2-10-1 (4)	0-0-0 (0)	0-0-0 (0)
1/4,000	11-14-7 (11)	30-16-26 (24)	18-19 (18)	0-2-0 (2)
1/8,000	25-25-31 (27)	37-42 (40)	33-24-32 (30)	0-0-0 (0)
1/16,000	91-64-60 (72)	75-79-70 (75)	60-62-70 (64)	14-16-21 (17)
1/32,000	100-114-98 (104)	108-118-96 (107)	86-110-112 (102)	43-58-53 (51)
No interferon	106-99-96-90 (98)	104-108-106 (106)	108-98-108-104 (103)	106-96-87 (100)
Dilution of virus stock needed to obtain indicated plaque numbers	10^{-5}	$10^{-4.8}$	$10^{-4.8}$	$10^{-4.6}$
Reciprocal of titration end-point dilution	10,000	11,500	11,500	32,350

* Human leukocyte interferon; undiluted sample at 10^6 U ml⁻¹.

† Average numbers are given in parentheses.

In selected experiments we titrated various ratios of effector to target cells. Figure 2 shows that interferon-mediated enhancement of CML activity at 39 °C was more pronounced with high than with low ratios of effector to target cells. This was not so at 37 °C. When the cultures were assayed for CML activity at different times after initiation, the peak of activity was always found at day 6, regardless of the temperature of incubation or the presence of interferon. Thus trivial kinetic differences can be excluded as the explanation of our observations.

The lymphoblastoid human B-cell line Daudi has been shown to be highly sensitive to the growth-inhibitory effect of interferon¹³. Daudi cells (given by H. Strander and J. Zeuthen) were cultured in microtitre plates using RPMI 1640 medium with antibiotics and 10% fetal calf serum as described elsewhere¹⁴. Interferon was added to the cells at the beginning of cell propagation, and proliferation was measured by the uptake of ¹⁴C-thymidine or by counting viable cells on days 3–4 (ref. 14). In this system growth inhibition is not due to cell killing because it is reversible¹⁴. The relative inhibition of growth

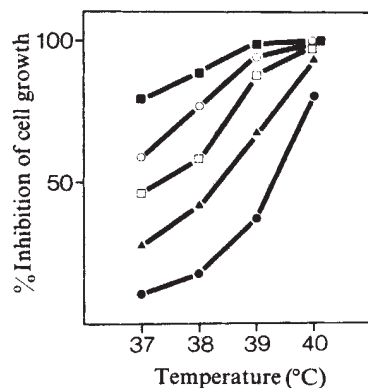


Fig. 3 Relative inhibitory effect of different interferon concentrations on the propagation of Daudi cells at different incubator temperatures. The results of quadruplicate cultures, each containing 2×10^4 Daudi cells in 0.2 ml of culture medium, with and without interferon in the doses were determined. DNA synthesis was quantified by addition of 0.02 μ Ci of ¹⁴C-thymidine per culture for the last 16 h of a 96-h culture period. Mean c.p.m. $\pm$ s.d. of the control cultures without interferon at the four temperatures were 20,340 $\pm$ 570, 26,890 $\pm$ 450, 26,370 $\pm$ 790 and 3,180 $\pm$ 190, respectively. The inhibition of thymidine uptake by a certain interferon concentration at a certain temperature has been calculated in percentage of the control at that temperature. ●, 1 partially purified human leukocyte interferon per ml.; ▲, 2 U ml⁻¹; □, 3 U ml⁻¹; ○, 5 U ml⁻¹; ■, 10 U ml⁻¹.

obtained by different concentrations of interferon at four temperatures is shown in Fig. 3. A pronounced increase in the inhibitory activity was found with increasing temperatures.

Our experiments show that the antiviral and two non-antiviral properties of interferon are temperature-dependent, but at different levels.

The antiviral effect was not increased until 40 °C was reached. Increase in temperature itself is known to inhibit viruses. This was overcome by adding more virus at the higher temperatures. The mechanisms by which interferon potentiates the alloreactive killer T-cells generated⁶ are unknown. Possibly interferon acts by inhibiting suppressor cells or by enhancing the expression of histocompatibility antigens on the cells¹⁵. The inhibition of the multiplication of Daudi cells (a B-cell line) might be analogous to the inhibitory effects of mouse interferon on B-cell functions *in vivo*⁵. We do not know whether a similar 'fever'-induced interferon-augmented generation of

killer cells and increased inhibition of growth occur *in vivo*. If so, fever during virus infections would potentiate host defence mechanisms. The fact that (1) the defence against viral disease is particularly dependent on cell-mediated immunity, (2) viruses are particularly good interferon inducers and (3) interferon is produced *in vivo* at elevated temperature leads us to suggest that interferon besides its antiviral role, is a normal immunoregulatory molecule leading the immune response in a 'more' cell-mediated direction at elevated body temperature.

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Errata

In the letter 'Electron microscopic location of protein thiol residues' by M. Stewart and V. Diakiw, *Nature* **274**, 184, line 21 in Fig. 3 legend should read: Tris-HCl, pH 8 at 4 °C. Samples for electron microscopy were . . .

In the review article 'Handedness of atoms and parity non-conservation' by G. Feinberg, *Nature* **271**, 509, line 11 on page 510 should read: approximately $|\delta| \approx 10^{-11}$.

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matters arising

Interpretation of short-period oscillations in ionospheric group heights

DEVLIN *et al.*¹ observed oscillations in the group height of reflections from a sporadic-E layer, with peak-to-peak amplitudes of about 200 m and periods around 10 s. As the sounding frequency increased, the oscillations were phase shifted in time and their period changed slightly. They ascribed these events to real oscillations in the reflecting surface possibly due to sound waves or an instability. Here we show that an interference mechanism is more likely.

The results indicate a reversal in phase as the sounding frequency changes by about 0.2 MHz. As the plasma frequency increases by about 1 MHz in 100 m the vertical wavelength of any wave motion is about 40 m, and the corresponding vertical trace velocity is 4 m s^{-1} . Devlin *et al.* assert that there is only one reflection point. This implies that the radius of curvature of the reflector is greater than the reflection height. Equating the wave amplitude to the group-height oscillations makes the horizontal wavelength more than 20 km, and the horizontal trace velocity more than 2 km s^{-1} . Clearly, a wave interpretation requires an almost vertical wave normal and a phase velocity of about 4 m s^{-1} ; this eliminates sound waves as a possibility. We suggest that instabilities with these characteristics are also extremely unlikely because these must travel either with the electron drift due to electric fields and thus at right angles to the field, or with the ion drift which is nearly horizontal. Both drift velocities would be expected to be some tens of m s^{-1} .

To show that the oscillations are due to interference effects, consider two echoes from group heights h' and $h' + \Delta h'$ where $\Delta h'$ changes with time but is so small that the radar pulses overlap. The two echoes at any frequency f interfere to give a resultant signal whose phase ϕ may be calculated. If the usual formula $h' = (c/4\pi)(\partial\phi/\partial f)$ is applied to the resultant signal, the apparent group height is given by

$$h'_{\text{app}} = h' + \frac{\Delta h'(1 + R \cos \theta)}{1 + R^2 + 2R \cos \theta} \quad (1)$$

where R is the ratio of the two echo amplitudes, and $\theta = 4\pi \Delta h' f/c$ is their

phase difference. Devlin *et al.* calculated $\partial\phi/\partial f$ using two frequencies 200 kHz apart, so we have done the same in Fig. 1. This plots h'_{app} against time for various frequencies, taking $R = 2.5$ and $\Delta h' = 0.007t \text{ km}$. Many features of the results of Devlin *et al.* are reproduced. The phase shifts result from a slight decrease in the period of oscillations as the sounding frequency increases which, in turn, is due to a more rapid interference cycle at the smaller wavelengths. The plot has the appearance of beating waves but this is due to the method of analysis. Strict use of equation (1) gives continuous oscillations of considerably larger amplitude. If a third echo is included, it is possible to model the results even more closely. The phase deviations extend no more than $\pm 24^\circ$, so that the apparent phase height variations are very small. Devlin *et al.* believe that there is only one point of reflection but we suggest that two echoes with a difference in group heights less than 800 m could not be resolved in the synthesised pulses they present which have lengths considerably greater than the 293 m quantisation level mentioned.

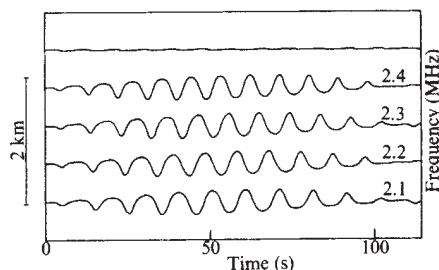


Fig. 1 Model variations of apparent group height at several frequencies and phase height (top trace) at 2.4 MHz for conditions described in the text.

Figure 2 shows a result from the University of Queensland phase ionosonde which has four receiving aerials situated approximately at the four corners of square of side 140 m. The time variations of group height recorded from each aerial at a frequency of 3.75 MHz are shown with the variations of phase height recorded from one of the aerials. The four group-height variations are similar and there are time shifts up to 2 s between the recordings. This is typical of an interference-induced effect, whereas the time shifts would be a small fraction of a second if the horizontal velocity of the wave motion were 2 km s^{-1} . The

variations of phase height are very small and have values of the same order as those predicted by the interference model.

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DEVLIN *et al.* REPLY—Short period, small amplitude oscillations observed in group height were claimed in our paper¹ to be associated with a sporadic-E layer being observed. It was also suggested that the actual phenomenon responsible may be sound waves or waves associated with plasma instabilities. Munro and Whitehead² agree that the observations were produced by a real ionospheric effect, but they suggest that the oscillations in group height are not a direct result of oscillations in the ionosphere, and are due to interfering echoes (from an undefined irregularity structure in the ionosphere).

Munro and Whitehead first reject our explanation on the basis of characteristics of possible waves indicated by the data. However, the results depend very much on the assumptions made. For example, the results indicate that the phase reverses within a frequency change of 0.2 MHz, but this is a very approximate value and it could be closer to 0.1 MHz. Similarly it is equally valid to assume that the plasma frequency changes by 1 MHz in 50 m rather than 100 m. The ionosonde steps 0.1 MHz in 0.02 s, so that with the above assumptions, waves with apparent vertical velocities close to multiples of 200 m s^{-1} will have very slow apparent vertical velocities. For the assumptions made by Munro and Whitehead the same effect occurs for vertical velocities near 500 m s^{-1} . Other reasonable assumptions give intermediate values so that almost any reasonable vertical trace velocity could give a small apparent velocity.

In deducing an apparent horizontal wavelength, Munro and Whitehead ignore any retardation effects. However, these may be important in the E-region when such small changes are being

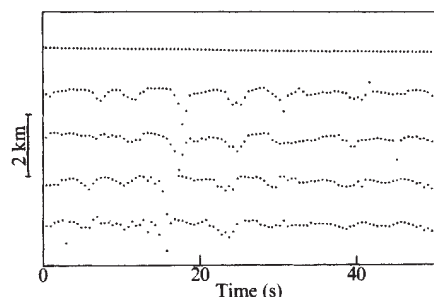


Fig. 2 Apparent variations of group heights recorded at four aerials and the corresponding phase height variations (top trace).

considered and especially when the data show unequal changes in group and phase path. Oscillations in the group path show changes of the order of ± 200 m while inspection of phase path records show the oscillations to be of the order of ± 10 m. Other effects such as Doppler shift due to atmospheric winds can also affect the apparent horizontal velocity. Consequently, it is difficult to confidently deduce trace velocities from the data.

Second, Munro and Whitehead consider that if the oscillations represent real changes in group height, then corresponding changes in phase height would occur and that phase height experiments would have previously detected such waves. However this argument ignores the possibility of retardation effects which can be important. Calculations of the effects of irregularities on group and phase path show that changes in phase path can typically be about a tenth of the change in group path^{3,4}.

Finally, if the effect is produced by interfering echoes, there is an unusual effect in that the oscillations are restricted to a relatively limited range of frequencies. Although this does not preclude interference as an explanation of these oscillations, it suggests that the interference mechanism will not be simple in nature. If the irregularities producing interference were moving vertically at the speed assumed by Munro and Whitehead, their progression through the layer should have been observed in Fig. 1. If the irregularities move horizontally, velocities of up to 300 m s^{-1} are implied which again means that sound or gravity waves might be involved.

It should be noted that the definition of the quantity θ used by Munro and Whitehead is strictly only valid for a mirror reflector and the more general definition is $\theta = 4\pi\Delta h_p/c$.

Thus it seems that although interference may be the explanation there are problems with the simple interference approach, but more importantly, invoking interference does not preclude the problem of identifying the type of irregularities responsible.

Clearly more work needs to be done to satisfactorily explain the oscillations and further studies are being carried out at La Trobe University.

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Swimming rats and human depression

WE believe that the rat swimming test of Porsolt *et al.*¹ is a valuable contribution to drug screening methodology and we are now using it in our present work. Sprague-Dawley rats are individually tested in a narrow plexiglass cylinder (height 40 cm, diameter 18 cm) containing 15 cm of water. Initially, the rats swim vigorously but later make only the movements necessary to keep their heads above water. In drug screening, naïve animals are given a 15-min swim on the first day, then dried and given the first of three spaced drug injections. They undergo a 5-min swimming test 24 h later in which the period of relative immobility is timed. As clinically effective antidepressant drugs are selectively identified in this way, Porsolt *et al.* conclude that the behaviour evaluated represents 'lowered mood' and 'despair' in the animals. We believe this interpretation is contrary to other behavioural activity seen in the test which they do not report.

We replicated their control group condition for the equipment described, strain used (167–230 g), most common subgroup size ($n = 5$), water temperature and procedure. We also made further observations (adjusted for time) comparing day 1 and day 2 tests. We confirm their finding with regard to the behavioural change seen but wish to describe it more fully. The rats quickly learn to touch bottom with their tails and hind feet. They are then able to maintain a position in which they prop themselves against the side of the cylinder without the energy expenditure required in swimming. This we consider to be an adaptive response.

We make the following points: (1) implicit in the two-day test is the idea that behaviour may change in the absence of any other independent variable. Early in the day 1 test, two indicators of behavioural disturbance were seen: diving^{2–4} and headshaking. Four of our subjects dived on the first day, none on the second. All showed headshaking on day 1, only one did so on day 2. Again, we interpret these changes as being adaptive in this situation. (2) If the immobility response represents 'despair', it should be maintained once adopted, that is, if the animal has given up, it will not try again. Instead, we found that all animals switched back to swimming and back again to immobility on both days. The count for such changes in the 5-min interval was: day 1, $\bar{x} = 3.6$; day 2, $\bar{x} = 3.8$; $t_{\text{dep}} = 0.25$. (3) Finally, emotional defaecation has been used as a measure of fearfulness in a variety of experimental contexts^{5–7}. Bolus counts for our subjects gave these results: day 1, $\bar{x} = 5.6$; day 2, $\bar{x} = 3.0$; $t_{\text{dep}} = 2.23$, d.f. = 4, $P < 0.05$ (one-tailed test). This supports the idea that having been rescued on day 1, the rats were less fearful on day 2.

We conclude that the animals are making adaptive responses to a stressful situation. Drug effects which reduce immobility and increase swimming time may predict which new antidepressant drugs will be effective. Such a result will be more convincing when concomitant behaviour is also observed and reported. Even then, such a result should be interpreted cautiously. The swimming test does not provide a model resembling depressive illness in human beings.

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PORSOLT AND JALFRE REPLY—The description of the rat's behaviour provided by Hawkins *et al.* corresponds well with what we have observed ourselves^{1,2};

the rats on first exposure to the apparatus show highly active swimming, headshaking, diving and defaecating frequently. With prolonged exposure, and particularly during the 5-min test on the second day, the vigorous activity declines, being interspersed with phases of immobility of increasing duration.

We do not, however, agree with their conclusion that the rats are merely making an adaptive response to a stressful situation which is unrelated to a state of lowered mood. Although it is evident that the rats do adapt to the situation in that their initial agitated behaviour rapidly subsides, we see no reason to exclude *a priori* the possibility that they may at the same time also be feeling depressed. Three lines of evidence would support this latter proposition. First, it is known that exposure to inescapable traumatic situations can induce symptoms of depression in man and higher animals^{3,4}. Second, our results show that a variety of clinically effective antidepressant drugs selectively decrease immobility whereas other drug classes do not^{1,2}. Finally, we have shown that immobility is also reduced by nonpharmacological treatments such as electroconvulsive shock and deprivation of REM sleep², procedures which are generally accepted to be effective in the treatment of human depression^{5,6}. A further point with which we disagree is the assumption of Hawkins *et al.* that for immobility to represent despair, it should be maintained once adopted. It seems to us more reasonable to expect the animal to oscillate between attempts to escape and immobility, with immobility predominating as the inescapability of the situation becomes more apparent, which is what both we and Hawkins *et al.* observe.

In general, we do not claim to have produced a model of human clinical depression in the rat. Nonetheless, our results encourage us to believe that our procedure provides an animal model of at least some aspects of depressed mood which is readily amenable to experimental manipulation.

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Effect of osmolarity on quantal size

VAN DER KLOOT reports¹ that he was unable to detect an amplitude change in miniature end plate potentials as a result of the imposition of osmotic changes in the incubation medium of the nerve-muscle preparation. He argues that his failure excludes a release mechanism depending on the free acetylcholine (ACh) in the cytoplasm because the change in osmolarity should have changed the terminal volume which should then have changed the free ACh concentration. This would have been then detectable as a change in the amplitude of the miniature end plate potential.

Unfortunately, he did not measure the change in the terminal volume so we have no idea how successful his procedure was in overcoming the various compensatory mechanisms for maintaining cell volume.

More critically, he neglected to measure the free ACh concentration. The factors which control this are unclear but it is certain that the concentration of free transmitter can change significantly in response to functional demands within five seconds². The activity of choline acetyltransferase in neuromuscular junction terminals is sufficient in appropriate conditions to synthesise (or degrade; it is fully reversible) the whole transmitter store within the 20-s period it took him to change the osmolarity and repenetrate the muscle³.

I am afraid we are no further forward unless Van der Klot can supply direct evidence that the free ACh concentration changed in the way he thinks it did.

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VAN DER KLOOT REPLIES—The changes in the volume of frog motor nerve terminals in hypertonic solutions were described by Clark¹. The nerve terminals surely shrink appreciably in hypertonic solutions.

The experimental demonstration of a swift mechanism for the homeostasis of free acetylcholine in nerve terminals, operating over massive changes in concentration, like that proposed by Marchbanks, would be of substantial interest in itself and certainly, as I pointed out in my report, could account for the observations within the framework of the gated channel hypothesis for quantal acetylcholine release. The major problem that would remain is how the

diffusion of charged acetylcholine cations through membrane channels could be independent of the potential across the membrane.

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Use of ADTN to define specific ³H-spiperone binding to receptors in brain

THE report of Leysen *et al.*¹ concerning the binding of the relatively new radiolabel ³H-spiperone to both dopamine (DA) and 5-hydroxytryptamine (5-HT) receptor sites suggests that caution is needed in using this radioligand for DA receptor binding

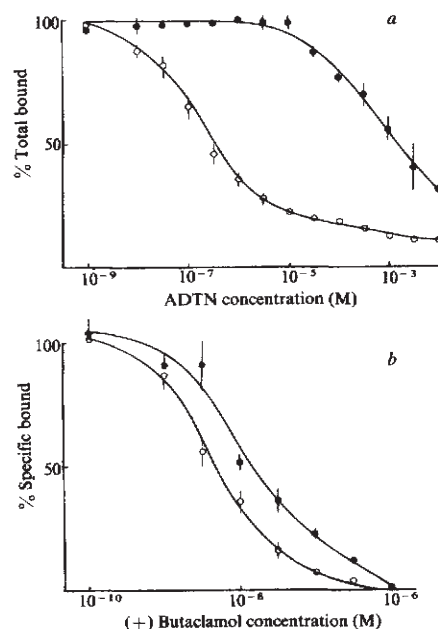


Fig. 1 Effect of ADTN (a) and (+)-butaclamol (b) on the binding of ³H-spiperone in rat brain striatal (O) and medial frontal cortex (MFC) (●) homogenates. Tubes contained 0.50 nM and 0.25 nM ³H-spiperone for the medial frontal cortex (MFC) and striatum, respectively, these represented the K_d values of saturable binding sites in the two brain regions. Each point represents the mean $\pm$ s.e.m. of 4-10 determinations from 2-5 separate experiments. In a, the results are expressed as % of total ³H-spiperone binding, corrected for filter blanks. In b, results are expressed as % of 'specific' ³H-spiperone binding, defined as that displaced by 1 μ M (+)-butaclamol.

Table 1 Inhibition of saturable ^3H -spiperone binding to rat medial frontal cortex or human frontal cortex and rat striatal or human caudate membranes by drugs

Compound	IC ₅₀ (nM)		Ratio $\frac{S}{FC}$
	Striatum (S)	Frontal cortex (FC)	
5-HT-related (rat)			
Tryptamine	82,000	31,000	2.6
5-Hydroxytryptamine	80,000	12,000	6.7
Methysergide	77	8	9.6
Quipazine	120,000	2,600	46.1
Cinanserin	3,700	54	68.5
DA-related (rat)			
ADTN	230	930,000	0.0002
Piribedil	47	29,000	0.002
Dopamine	5,800	415,000	0.01
Haloperidol	5.6	190	0.03
(+)-Butaclamol	6.6	46	0.14
Bromocriptine	36	160	0.23
(-)-Butaclamol	900	3500	0.26
α -Flupenthixol	2.8	9.0	0.31
DA-related (human)			
ADTN	375	>100,000	>>0.0003
(-)-Butaclamol	1,800	130,000	0.01
(+)-Butaclamol	0.9	28	0.03
Dopamine	60,000	1,500,000	0.04

Membrane preparations of rat brain regions (dissected according to the procedure of Glowinski and L.L.I.¹⁰ and Emson and Koob¹¹ were obtained as previously described²⁻⁴. Tubes containing 50 μl ^3H -spiperone (Radiochemical Centre; specific activity 26 Ci mmol⁻¹), 50 μl test drug and 900 μl homogenate (equivalent to approximately 5 mg wet weight of original tissue) were incubated for 10 min at 37°C. Membranes were then collected by filtration, washed and the bound radioactivity determined²⁻⁴. The concentration of each drug required to inhibit specific binding of ^3H -spiperone by 50% (IC_{50}) was determined by log-probit analysis of experiments such as those shown in Fig. 1. The concentration of ^3H -spiperone was 0.25 nM for rat striatum or human caudate and 0.5 nM for rat medial frontal cortex and human frontal cortex (Brodmann area 10). Nonspecific binding, defined as the ^3H -spiperone binding observed in the presence of an excess of nonradioactive spiperone (1 μM), was subtracted and represented approximately 10% of total binding in the rat and 18% in the human in both regions. For each drug inhibition of ^3H -spiperone binding was measured in duplicate or triplicate at 4–8 concentrations. Ratio S/FC is the ratio of the IC_{50} in striatum to frontal cortex. Total binding of ^3H -spiperone (corrected for filter blanks) was 65 ± 2 and 97 ± 1 fmol per mg protein in medial frontal cortex and striatum, respectively (mean $\pm$ s.e.m., $n = 8$ to 12 determinations).

studies²⁻⁵. We can support their conclusion concerning the heterogeneity of ^3H -spiperone binding sites and suggest a way of defining the DA component of ^3H -spiperone binding. In most studies, the potent neuroleptic drug (+)-butaclamol with its pharmacologically inactive enantiomer (-)-butaclamol has been used to define 'specific' binding of ^3H -spiperone and other ligands to central DA receptors. However, as butaclamol also has potent and stereoselective effects on ^3H -LSD binding to central 5-HT receptors⁶, the stereospecific binding as defined by the difference between (+) and (-)-butaclamol is likely to include both DA and 5-HT receptor components. We have found that 2-amino-6,7-dihydroxy-tetralin (ADTN), a rigid dopaminergic agonist⁷⁻⁹, displaces ^3H -spiperone selectively from areas which have a rich dopaminergic innervation. ADTN may, thus, be a more suitable agent for defining specific binding of radioactive ligands to central DA receptors.

Saturable binding of ^3H -spiperone⁴ could be detected not only in dopamine-rich brain regions, such as rat striatum

and human caudate nucleus, but also in areas containing only small amounts of endogenous DA, such as rat medial frontal cortex and human frontal cortex (Brodmann area 10). The density of saturable ^3H -spiperone binding sites in the rat striatum was only about 1.5 times higher than in medial frontal cortex.

The displacement of ^3H -spiperone by nonradioactive DA or 5-HT in homogenates of rat medial frontal cortex and striatum was markedly different (Table 1). DA (and related compounds) was more potent than 5-HT (and its congeners) as a displacer of ^3H -spiperone binding in the striatum, but in the medial frontal cortex the potencies were reversed. The greatest difference in affinity for binding sites in basal ganglia and frontal cortex was observed with ADTN, which had an affinity for basal ganglia receptors some 4,000 times greater than that for binding sites in medial frontal cortex in rat brain (Fig. 1a), with an even greater difference between human caudate nuclei and frontal cortex (Table 1). The differences in drug sensitivity of ^3H -spiperone binding in basal ganglia and

frontal cortex suggest that the drug binding observed in frontal cortex may largely represent an interaction with 5-HT receptors, whereas in basal ganglia tissue ^3H -spiperone binding occurs predominantly to DA receptors.

Spiperone, and certain other neuroleptic drugs, apparently interact with high affinity at both DA and 5-HT receptors in the CNS; our estimates of K_d values for ^3H -spiperone binding in rat striatum ($K_d = 0.25$ nM) and medial frontal cortex ($K_d = 0.5$ nM) indicate only a small difference in the affinity of spiperone binding at these different sites (DA and 5-HT); in human tissue the K_d for these two areas is, in fact, the same ($K_d = 0.5$ nM). Similarly, as reported previously, the neuroleptic drug butaclamol interacts potently and stereoselectively at both these sites. Figure 1b shows that specific binding of ^3H -spiperone, defined as that displaced by 1 μM (+)-butaclamol, is detectable in homogenates of both striatum and medial frontal cortex although (+)-butaclamol is slightly less potent in inhibiting ^3H -spiperone binding in the cortical preparations. Thus, the use of the isomers of butaclamol to define 'specific' binding of ^3H -spiperone exclusively to CNS DA receptors would clearly yield misleading results.

The highly selective displacement of ^3H -spiperone from its binding sites in striatum by the DA agonist ADTN, makes this drug the most suitable for defining DA receptor binding in rat and human brain.

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reviews

Products of creative imagination

Horace Barlow

The Origins of Knowledge and Imagination. By Jacob Bronowski. Pp. 146. (Yale University Press: London and New Haven, Connecticut, 1978.) £5.75; \$7.95.

THIS short and very readable book consists of the six Silliman Lectures delivered at Yale in 1967. Bronowski apparently intended to publish it with a companion volume on the nature of man's artistic endeavours, but the preparation of his television series *The Ascent of Man* delayed this, and it had not been done when he died in 1974. The present book therefore has to stand alone, and it does so very satisfactorily if one regards it as an essay in constructive speculation, rather than as a scholarly work that compels ascent by weight of evidence.

His programme is an ambitious one, for it is "to ask what our knowledge of the external world rests on when we think of ourselves, not as having God-given insight into it, but as being human animals penetrating it with the physical gifts we possess". As he says, this was Kant's programme in the 1760s, and he thinks it is high time to carry it through. Of course, he does not succeed; how could he in 135 pages? But in these six lectures he utters a good many provocative and probably insightful remarks.

He starts by pointing out that man's physical gifts include a well developed visual system, and the very words imagination, visionary, foresight, insight, show how man's important mental gifts are conditional upon his sense of sight, his use of the optical image in his eye. He then goes on to talk about language, contrasting the emotive, message-giving properties of animal communications with the sentences of human communication in which the individual words have separate meanings. "In the beginning was the word" is exactly wrong, he says: in the beginning was the sentence, and separable, recombinable, words are man's great invention. In this chapter he recounts some nice snippets of information about animal language, such as Fabre's account of the grasshoppers who chirp just before they hop; if they were to fail to give their goodbye chirp, all the other grasshoppers would be terrified by the sud-

den movement of their hop, as they are by any other sudden movement in their surroundings. But our respect for animal communication has grown recently, and though the fact that words have separable meanings is important, Bronowski's most original and visionary remark in this lecture may well be, "Nothing that I say today will be right in ten years' time."

In the third lecture he compares Newton's " $G = km_1m_2/r^2$ ", and Blake's "A robin red-breast in a cage/Puts all heaven in a rage". Both, he claims, are products of man's creative imagination. It was this same power that broke the words out of the emotive, message-giving proto-sentence, attached separate meanings to them, and thus allowed them to generate new sentences carrying new and different messages by the process of reordering and recombination. In the examples of this chapter Newton's imagination led him to visualise the apple, not falling passively, but being thrown faster and faster until it orbited the earth, and he linked the apple's imagined orbit with the circling moon. Thus, he could isolate and name in a "word" of his language, $G = kmm/r^2$, an astonishing range of precise phenomenon. He does not go into Blake's "word" nearly so deeply, perhaps because of his planned book on the artist's endeavour, but he points out the infallibility with which the two lines convey a rather exact, isolatable, meaning to every reader.

This chapter is the core of the book, and the suggested analogy between the separable word and the message created by poet or scientist is clearly an interesting thought. But I have two complaints about it, a sin of omission and a sin of commission. The omission is that he does not really show how linking the apple and the moon isolates the law of gravity. Perhaps it is a bit like attaching a name to an object or class of objects, for you have to identify the class as worthy-of-naming before you attach the name; but there is more to it than this and he has not quite put his finger on it. Perhaps he did not have the time or space, but in that case his sin of commission is aggravated. This sin is name-dropping; in this single chapter, after Newton and Blake, he mentions Wittgenstein,

Karl Pearson, Kenneth Craik, Einstein, Piaget's work but, curiously, not his name, Olbers, Hermann Bondi, Glauber, Humphrey Davy, Boltzmann, Bentley, Adams and Leverrier, Max Planck, Harvey, Galen, and Kepler. I am glad to say he also mentions Jehovah early in the chapter, but needless to say he cannot really do justice to them all; they are mere beads on a rosary, and if I were his priest I would give him no forgiveness.

In the remaining chapters he goes more deeply into the nature of scientific laws, though to my mind he does not rectify the sin of omission for he does not go back to further discussion of that almost magical step, the linking of apple and moon that named and circumscribed gravity.

The fourth chapter revolves around Epimenides the Cretan who said, "All Cretans are liars", and it contains an interesting discourse on Russell, Gödel, Tarski, the problems of self-reference, and the limits of consistent axiomatic systems. I find it difficult to get a very precise message from this chapter, but I think he is saying that, on the one hand, the interconnections in the real world are richer and more complex than we can ever know, whereas, on the other hand, axiomatic systems turn out to be surprisingly restricted in their capacities; therefore one should pick the axiomatic system that best matches the patterns of connectivity you know about, but one must be prepared to change it. That sounds almost too full of commonsense for a theoretician.

The last two chapters are less concerned with the abstract mathematical apparatus of science, more with how it works and makes progress; that is, it makes mistakes and then corrects them. The final chapter is on the ethics of science and smacks a bit of a puff for the scientific community: we are truthful, we rely on the truthfulness of other scientists, and look at the glories that truthfulness has led us to. One response to this might be, "I am looking, but I'm not sure how glorious it is". Another, from a non-scientist, might be, "Of course you scientists trust each other and have formed a mighty powerful gang. But you are not the first such gang in history, for even the most corrupt politician trusts some, and is trusted by some. I'll wait and

see how you behave when times get rough, when the money runs out. And anyway, tell me please, why should I trust you and believe that you are being truthful to me?" I don't think Bronowski would convince such a sceptic, who would of course be delighted by the revelations of Watson's *The Double Helix*, and also by some of the specious pleas made by scientists in recent years when the money has been running out. Nevertheless, those who practice science will recognise that much of what Bronowski says is

right, though I think it has to be said modestly and quietly, for a big mouth never sounds truthful.

These are interesting lectures and they are eminently enjoyable to read, with a good story or 'bon mot' on every page. Because they are so light and readable, the true value of Bronowski's thoughts and speculations may easily be underestimated. □

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Eye movement during vision

Movements of the Eyes. By R. H. S. Carpenter. Pp. 420 (Pion/Academic: London, 1978.) £13.50; \$27.

A MOMENT'S thought makes it clear that we collect sense data by active exploration of the world with our receptive surfaces, including the retinas of the eyes; the resulting relationships between the movements of the eyes and visual perception presents fascinating and formidable problems. Yet the movements themselves are particularly simple ones, as they engage a system of constant inertia, the eyeball and its muscles; eye movements need not involve the postural adjustments required by limb movement and are confined to rotations without translation. Thus, they ought to offer an ideal system on which to study many principles of the control of movement.

Dr Carpenter's book deals with the whole subject of eye movements, their control and their relationship to vision, and does it very well. The advantages for the research worker of its comprehensiveness, may, unfortunately, deter the medical or biological undergraduate; it is not a book which can quickly be scanned to provide a superficial view.

The first of three sections describes the types of eye movement found in man; there are six: the compensatory vestibulo-ocular responses which keep the image steady on the retina, tracking movements, saccades (used to change fixation from one object to another), vergence movements (which train the two eyes on the same target), and miniature eye movements (which may be the results of 'noise' in the control system but without which the stabilised image rapidly fades from perception).

The second part contains a particularly clear and comprehensive account of the static and dynamic properties of the globe and its muscles, which together form a highly damped

system the force/displacement properties of which are remarkably linear, and ends with descriptions of the afferent and efferent connections of the oculomotor system. The final section deals with the relationship of eye movements to visual sensation and with models of the control systems for the various kinds of movements.

The first of two appendices is a useful summary of techniques of measuring eye movements; the second is a brief introduction to linear systems analysis. There is a good deal of emphasis on linear systems analysis throughout the book and Dr Carpenter has succeeded in his declared intention of presenting this in a way which should not alarm the non-mathematician. The mathematical appendix deals with a surprisingly large amount of material; surprisingly large, as it contains not a single differential equation. Although it will allow the uninitiated to follow this book, which relies on the 'differential operator' and eschews the Laplace representation, I fear that it will not equip him to cope with most of the original papers which do neither.

In addition to describing how elegant models can be built to describe the behaviour of the control systems, Dr Carpenter deals very fairly with the major problem of identifying, within the complexity of the nervous system, the physical mechanisms which may populate the black boxes; it is not surprising that the results are not very satisfying when the neural mechanism of even such a fundamental component as the integrator required by all models is elusive.

One may be forced to echo the final conclusion that "sadly, . . . nothing in the brain is as simple as it seems". But it is also true that we now have a much better account both of what we know, and of what we don't know, about the oculomotor system. For this we are in Dr Carpenter's debt; his book will be useful to all who are interested in control of movement or in vision.

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Computer software

Problems, Programs, Processing, Results: Software Techniques for Sci-Tech Programs. By P. Quittner. Pp.381. (Adam Hilger: Bristol, Academic: Kiado: Budapest, 1978.) £13.50.

THE author's intention, as expressed in the preface of this ambitious book, is to provide information about the workings of computer systems so that users may exploit available facilities more efficiently. Unfortunately, the title is somewhat misleading, as it implies an exposition of methods for designing and writing computational software; whereas by far the largest part is devoted to machine architecture, systems software, operating systems and data organisation.

Spot checks on how scientists use computers show that many expensive resources are squandered through clumsy programming. Of course, scientists are concerned with obtaining results quickly not with winning Nobel prizes for elegant software. However, it is surprising how a little knowledge of what happens to programs as they go through the machine can not only improve efficiency but also make the programmer's life easier.

This book will be of value to those seeking such knowledge. It covers a formidable number of topics in a clear and well-organised manner, although there is some imbalance in the depth of treatment given to some areas at the expense of others. It is doubtful, for example, whether a complete table of machine instructions for the IBM 370/145 should be included in a general book when there is no mention of structured software design concepts.

The breadth of the work may be judged from the fact that its 380 pages cover languages, assemblers, compilers, loaders, program testing, filing schemes, database management and operating systems. Specialist computer scientists may find its treatment of individual subjects too concise, except as introductory material and a stimulus to further reading for which a comprehensive bibliography is provided.

The book was written by a Hungarian with a refreshingly lucid command of English and is a welcome example of a textbook published as a joint venture between East and West.

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Fungal development

The Filamentous Fungi. Vol. 3: Developmental Mycology. Edited by J. E. Smith and D. R. Berry. Pp. 464. (Edward Arnold: London, 1978.) £24.50.

OVER the years, the attractive and often bizarre range of morphological forms produced by filamentous fungi have proved irresistible to individuals ranging from illustrators of the earlier mycological texts to, more recently, overly self-indulgent scanning electron microscopists. The attractions have not gone unnoticed by developmental biologists, a band whose ranks have recently been swelled by molecular biologists ruefully contemplating the day when *Escherichia coli*, with a final intake of its terminal electron acceptor, yields its last molecular secret. And this is where a basic problem lies. The molecular biologist is superbly equipped to study the molecular basis of development in biological systems, such as *E. coli*, which are easy to manipulate in the laboratory. But filamentous fungi are anything but easy to work with in the laboratory. Attractively shaped differentiated structures they certainly produce, but isolating fractions rich in these structures, a necessary preliminary to a study of their molecular architecture, is often virtually impossible. Nevertheless, the molecular basis of differentiation in filamentous fungi has for many years had its coterie of practitioners. *Developmental Mycology*, the third volume in a series entitled *The Filamentous Fungi*, is a progress report up to and including 1976 on the activities of this cavalier band.

The editors took a very broad view of the term 'fungal development', taking license to include hyphal growth. This proved to be a bonus for, in molecular terms, this is undoubtedly the best understood aspect of the subject. At the same time, they have adhered strictly to filamentous fungi, with the result that a discussion of differentiation in the cellular and acellular slime moulds is omitted.

In all, there are 22 chapters, the first third of the book containing chapters on various aspects of hyphal growth. The chapters on fungal protoplasts and on mathematical analysis of growth are highly recommended, and both make a valuable contribution to the literature. But the dust jacket on the book tells us that the text aims to provide an integrated perspective of fungal development. Why then did the editors commission separate chapters on the cytology and enzymology of hyphal tip growth? Both are good accounts, but one chapter bringing

together both aspects of the subject would surely have more long-term value.

The middle section of the book deals with fungal developmental processes that lead to formation of structures that are morphologically different from hyphae. This section starts with a very readable chapter on yeast-mycelium dimorphism. Admittedly, the authors were able to call on a large body of experimental data but, by concentrating on fundamental concepts, they have produced a most valuable article. The balance between cytology and biochemistry that one would ideally like to see in the remaining chapters is, however, all too obviously missing. In general, this is no fault of the authors, but (as already outlined) results simply because the systems being studied are not amenable to sophisticated biochemical examination.

The book ends with chapters on fungal development induced by light and temperature, circadian rhythms, cell ageing and autolysis, cytoplasmic inheritance and senescence and fungal development and metabolite formation, reflecting yet again the broad view of developmental mycology taken by the editors. Although some of these subjects, such as production of secondary metabolites, have recently been thoroughly reviewed elsewhere, others including circadian rhythms and cell ageing have not, and so are exceptionally welcome.

Cerebral potentials

Progress in Clinical Neurophysiology. Vol. 3: Language and Hemispheric Specialization in Man: Cerebral Event-Related Potentials. Edited by J. E. Desmedt. Pp. 286. (S. Karger: Basel, 1978.) SFr./DM98; \$43.75.

A PROMINENT problem in neurobiology is to specify the properties of the speech systems of the human brain. Why are they alone capable of elaborating speech and language? We do know that it is not merely on account of their inflow and outflow; the comparable region of the 'subordinate' or non-speaking cerebral hemisphere has the same connexions, yet that region is involved in sustaining language only up to 10-14 yr of age and cannot thereafter become involved. We do not know why language generally resides in only one cerebral hemisphere; why this should be the left hemisphere; nor yet why language develops readily in human children but only partially and after laborious training (if at all) in apes.

The publishers make the sweeping claim that the contributions to the book "go beyond the standard review-type article by developing concepts in each area of Developmental Mycology". Some, particularly those that describe aspects of hyphal growth, do, whereas others do not and indeed could not because of the paucity of information available on many differentiation processes. When one turns, however, to other biological developmental processes, particularly those in prokaryotic microbes, one has seen over the years the emergence of fascinating, if at times contentious, new concepts such as commitment, long-lived mRNAs and regulation through modification to enzyme structure. *Developmental Mycology* would have been richer as a text if these concepts had at least been introduced, and their possible role in fungal development speculated on. However, the only attempt at an introduction to basic concepts is a tiny six-page chapter which does scant justice to the concepts of differentiation.

But these are not major shortcomings. *Developmental Mycology* is a welcome and timely volume which should be available to all who work with filamentous fungi, and not just those whose interests are in fungal development.

A. H. Rose

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The majority of the more than 30 contributors to this book are only peripherally concerned with the neural bases of language. They have described a variety of temporal relationships between the mass electrical potentials of the brain and linguistic or other performance. The potentials themselves, rather than the behavioural processes, seem to have become their main preoccupation. Indeed, new findings on the potentials are reported. Unfortunately the mass potentials afford an imprecise index of the electrical activity of the brain. Certainly they serve to lateralise language functions to one hemisphere, thus confirming the earlier clinical and experimental observations. And the more informative methods of recording from single brain cells cannot be applied to man. So we have to accept even a small step forward, one that is unlikely to reveal any truly fundamental new knowledge, with the reassurance that in the hands of these particular expert contributors the limits of the usefulness of this approach have now probably been reached.

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Geology of the Mediterranean basins and margins

The Ocean Basins and Margins. Vol. 4A: The Eastern Mediterranean. Edited by A.E.M. Nairn, W.H. Kanes and F.G. Stehli. Pp.503. (Plenum: New York, 1978.) \$59.40.

ONE might perhaps be forgiven for being surprised that the Mediterranean Sea should be included in a series with the title *Ocean Basins and Margins*, especially as some geologists would question whether any or all of the Mediterranean is underlain by true oceanic crust. However, earlier volumes in this series have concentrated on the geology of the land areas marginal to the oceans rather than the geology and geophysics of the basins themselves. A corollary of this seems to be that as the area of the ocean basin being considered decreases, and the proportionate length of the margins increases, so does the length of the treatment given. Thus, whereas the South Atlantic and the North Atlantic each commanded rather less than 600 pages, the Gulf of Mexico and the Caribbean warranted 700, and the Mediterranean receives over 900.

In fact, in this latest volume, the imbalance between geology and geophysics, and between the margins and the basins is largely redressed; and more so than previously the editors and publishers have achieved their objective of "presenting surveys of the relevant data, both geological and geophysical, to provide a broad overview of geological processes under and near the Earth's oceans". The main problem, it would seem, was an editorial one: how to marshal such a wealth of excellent material which was obviously too great for a single volume. Thus, despite its title, only half of the review papers in the present volume relate specifically to the Eastern Mediterranean; the four introductory chapters relate to the Mediterranean as a whole; and the last chapter reviews work on the Black Sea and Sea of Azov.

The volume opens with a particularly succinct and lucid overview, by Laubscher and Bernoulli, summarising the palaeogeography and tectonic evolution of the Mediterranean area

since the late Palaeozoic, and making the important distinction between the Palaeozoic 'Palaeotethys', and the Mesozoic 'Tethys', beloved of stratigraphers and sedimentologists. This is followed by a chapter by Hsü, who concentrates on the general tectonic evolution and geophysical characteristics of the Mediterranean basins, providing a useful, if at times speculative, summary of ideas.

Two lengthy and detailed chapters on geophysical data follow. The first, by Stanley, integrates continuous reflection profiling results and direct sampling to give a picture of post-Miocene sedimentation and tectonics, and the second, by Lort, reviews all geophysical data, basin by basin, blow by blow.

The remaining six chapters deal with the geology of the land areas marginal to the eastern Mediterranean, and fall conveniently into five regions, each of a comparable size: the area bordering the Adriatic, Greece and the Aegean,

Turkey, the Levant, and Egypt. The geology of Libya, it would seem, has been omitted, and that of Greece, the Aegean, and Cyprus gets rather brief and inadequate treatment.

I was left wondering why it is that the chapters are of such varying lengths, some rather unnecessarily detailed for reviews of this type, and others all too brief. I feel that had each author been kept to a word or page limit, a single and more uniform volume on the whole Mediterranean area could have been produced. Aside from this minor shortcoming, however, the volume is a worthy addition to the series, providing authoritative and comprehensive reviews which will constitute invaluable summaries and sources of references for many years to come.

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Plasma protein analysis

Plasma Proteins: Analytical and Preparative Techniques. By P. C. Allen, E. A. Hill and A. M. Stokes. Pp 254. (Blackwell Scientific: Oxford, 1978.) £11.75.

THE authors of this trim publication suggest that it will provide a useful manual for students embarking upon a project in plasma protein chemistry. However, prospective buyers ought to be advised that over half the text describes general analytical and preparative methods, which are probably already available to them in one or more standard works.

The section on analytical methods concisely describes electrophoretic, immunochemical and molecular weight estimation techniques in theory/practice format (105 pages). Serum proteins are used as examples to illustrate some of these techniques. Obviously, in practical texts like this, accuracy of description is of prime importance and there seems to be sufficient attention to this point. However, one toxic mistake appears on p12 where "50g of mercuric acid" is advocated in a recipe for a protein stain.

The preparative procedures section (41 pages) presents the classical Cohn fractionation of plasma, but the bulk of the text describes gel permeation, ion-exchange, affinity chromatography and protein concentration methods. Incidentally, quite a bit of this information is available free; as the authors state

it is "derived ostensibly from the operating instructions issued by various manufacturers".

On p160 the reader can get to grips with practical techniques for isolating specific plasma proteins. About 19 proteins including subclasses (for example, lipoproteins, immunoglobulins, and so on) are covered. Although the authors do not intend this section to be exhaustive, the lack of a description of the preparation of prealbumin is notable, as this is one of the very few plasma proteins of known function, sequence and structure. For the majority of the other proteins, there is a good short review, complete instructions for purification, and sufficient references to enable the original sources to be traced.

Collections of practical details for the isolation of proteins are often at the mercy of the original literature, which is notoriously uncritical. However, the authors rightly include more than one point of view where this is appropriate and point out where controversy exists about the homogeneity of a given protein.

There is certainly room for a publication which brings together the widespread literature on isolation of plasma proteins. The material in this book is well presented and very readable, but I feel that by giving such extensive coverage to general biochemical techniques it fails to do justice to the particular plasma proteins described.

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● *Introduction to Biological Scanning Electron Microscopy* by M. A. Hayat (for review, see *Nature* 274, page 98; 6 July 1978) is published in the UK by MTP Press: Lancaster.

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announcements

Appointments

Dr Anselm C. Dunham has been appointed to the Chair of Industrial Mineralogy at the University of Hull.

Professor A. E. Bender has been appointed to the Chair of Nutrition and Dietetics and Head of the Dept of Food Sciences and Nutrition at Queen Elizabeth College, University of London.

Appointments at the University of Manchester: **Professor Ian Douglas** to Professor of Physical Geography from 1 January 1979; **Mr Harold Fox** to Professor of Reproductive Pathology in the Depts of Pathology and of Obstetrics and Gynaecology.

Dr J. MacMillan has been appointed to a personal chair in Organic Chemistry at the University of Bristol from 1 August 1978.

The University College of Wales Aberystwyth has appointed **Dr Graham Williams** to a personal Chair in Physical Chemistry.

Brunel University announces the appointment of **Professor Roland Terry** as Head of the Dept of Applied Biology with effect from 1 October 1978.

The University of Newcastle upon Tyne has announced the following: **Professor J. D. Thornton** appointed Dean of the Faculty of Engineering for three years from 1 August 1978. **Dr John Simon Gilbert Miller** appointed to the Chair of Anatomy from 1 August 1978 and also to the headship of the Department of Anatomy for five years from 1 August 1978. **Professor D. H. Whiffen** appointed to Head of the School of Chemistry for five years from 1 October 1978. **Professor J. W. Thompson** appointed to Head of the Dept of Pharmacological Sciences for five years from 1 August 1978. **Professor F. Goodridge** appointed to Head of the Dept of Chemical Engineering for five years from 1 August 1978.

Meetings

28–31 August, **5th Canadian Symposium on Remote Sensing**, British Columbia (Dr Y. Jim Lee, Technical Program Chairman, c/o Pacific Forest Research Centre, 506 West Burnside Road, Victoria, British Columbia, Canada).

28 August–1 September, **4th International Biodeterioration Symposium**, Berlin (Professor Dr H. C. Günther Becker, Bundesanstalt für Materialprüfung, Unter den Eichen 87, F-1000 Berlin 45, FRG).

28 August–1 September, **86th Annual Convention of the American Psychological Association**, Toronto (American Psychological Association, 1200 Seventeenth Street, N.W., Room 500, Washington, D.C. 20036).

28 August–1 September, **8th International Colloquium on Plant Analysis and Fertilizer Problems** (R. L. Bielewski, Plant Diseases Division, DSIR, Private Bag, Auckland, New Zealand).

30 August–6 September, **4th International Congress for Virology**, The Hague (Congress Centre, PO Box 9000, The Hague, The Netherlands).

3–6 September, **Symposium on Mechanics of Wave-induced Forces on Cylinders**, Bristol (Dr T. L. Shaw, IAHR Symposium, Dept of Civil Engineering, University of Bristol, UK).

3–8 September, **Annual Congress of the British Veterinary Association**, Lancaster (Christine Nicholls, BVA, 7 Mansfield Street, London W1, UK).

4–7 September, **6th International Symposium on Medicinal Chemistry**, Brighton (Scientific Secretariat, 31 Plane Tree Way, Woodstock, Oxford, UK).

4–7 September, **1st International Topical Meeting on Muon Spin Rotation**, Rorschach (Conference Secretariat, c/o Schweiz. Institut für Nuklearforschung (SIN) CH-5234 Villigen, Switzerland).

4–7 September, **International Congress of Aerospace Medicine** (The Secretariat, Cavendish Medical Centre, 99 New Cavendish Street, London W1, UK).

4–8 September, **14th International Conference on Physics of Semiconductors**, Edinburgh (The Meetings Officer, Institute of Physics, 47 Belgrave Square, London SW1, UK).

4–8 September, **5th International Symposium on Living and Fossil Diatoms**, Antwerp (Dr John Barron, US Geological Survey, Menlo Park, California).

4–8 September, **International Symposium on Seed Protein Improvement in Cereals and Grain Legumes**, Neuherrberg (Georges Delcoigne, International Atomic Energy Agency, Kärlntnerring 11, PO Box 590, A-1011, Vienna, Austria).

4–8 September, **4th International Symposium on Physical Organic Chemistry**, York (Dr John Gibson, The Chemical Society, Burlington House, London W1, UK).

4–9 September, **IUIS Symposium—**

Course on Autoimmunity, Spetsai (Dr M. Raff, Zoology Dept, University College London, London WC1, UK).

4–9 September, **2nd European Neurosciences Meeting**, Florence (ENA Secretariate, PO Box 45, 2280AA Rijswijk, The Netherlands).

4–15 September, **Lasers in Atomic Physics**, Egham (Dr P. R. Knight, Physics Dept, Royal Holloway College, Egham, Surrey, UK).

6 September, **Water and Sediment Movement on the Continental Shelf**, London (K. R. Dyer, Institute of Oceanographic Sciences, Wormley, Godalming, Surrey, UK).

6–8 September, **Deposition and Filtration of Particles from Gases and Liquids**, Loughborough (The Conference Secretariat, SCI, 14 Belgrave Square, London SW1, UK).

8–18 September, **The Molecular Biology of Picornaviruses**, Maratea (Dr R. Perez-Bercoff, Institute of Anatomy, Gloristr. 19, 8006-Zurich, Switzerland).

9–12 September, **Science, Technology and Development**, Algiers (Professeur J.-M. Legay, 31 rue Barrier 69006 Lyon).

10–13 September, **Uranium—an International Assessment**, Las Vegas (Joel E. Jobst, EG & G Inc., PO Box 1912, Las Vegas, Nevada 89101).

10–13 September, **4th International Symposium on Contamination Control**, Washington (Mrs Betty Pearson, Institute of Environmental Sciences, 940 East Northwest Highway, Mt Prospect, Illinois 60056).

10–14 September, **Euchem Conference on Organic Free Radicals**, Cirencester (Dr John Gibson, The Chemical Society, Burlington House, London W1 UK).

10–16 September, **2nd International Congress of Entomology**, Ibadan (Director, Institute of Agricultural Research, and Training, PMB 5029, Moor Plantation, Ibadan, Nigeria).

10–16 September, **2nd International Congress of Ecology**, Israel (Conventions-Kopel, 122 Hayarkon Street, PO Box 3054, Tel Aviv, Israel).

10–22 September, **Humoral Immunity in Neurological Diseases**, Antwerp (Dr A. Lowenthal, Universitaire Instelling Antwerpen, Universiteitsplein 1, 2610 Wilrijk (Antwerp) Belgium).

11–12 September, **Enzymes in Industry and Medicine**, Exeter (Miss Sara Unwin, The Chemical Society, Burlington House, London W1, UK).